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FISH CELL DNA REPAIR AND BREAKAGE ASSAYS FOR ASSESSING AQUATIC GENOTOXICITY

by

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A Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF ZOOLOGY

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

October 1985

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Date Jan 29/86

DE-6(3/81)

ABSTRACT

It is claimed that many cancers in man are causally related to chemical carcinogen exposure. Similarly epidemiological studies of fish populations have associated an elevated tumor incidence with environments contaminated with chemicals, some of which have demonstrated carcinogenic activity. Sampling and pathologically screening large numbers of fish is labour intensive, costly, time-consuming, and field studies provide only circumstantial evidence for an etiological agent. A variety of quick and inexpensive assays have been used with mammalian cells to detect probable carcinogenic activity by analyzing for DNA-damaging events. More recently fish cells and metabolizing enzymes are being used in these assays in order to provide tests with more relevance to the aquatic environment, validate mammalian test results, determine fish cell sensitivity to genotoxic agents, and, ultimately, to develop techniques to directly assess the genotoxic:carcinogenic potential of a particular environment.

The present study initially compared the DNA repair response in fish and mammalian cells. Although the measured repair response of fish cells was found to be very low, in comparison to mammalian cells, the assay procedure was altered to increase the fish cell sensitivity. Despite being able to detect DNA repair in fish cells treated with 3,4-benzopyrene the repair technique was felt to lack the sensitivity necessary for *in vivo* monitoring. However, the low amount of repair measurable in fish cells suggested that tests for DNA

breakage may be more sensitive. Subsequent comparative experimentation with the chromosome aberration and micronucleus tests confirmed this. Treatment of fish cells with 3,4-benzopyrene resulted in chromosome aberrations. Polycyclic aromatic hydrocarbons like 3,4-benzopyrene are important aquatic contaminants and have been found in elevated concentrations in sediments from Sturgeon Bank, an area near Vancouver at which a sewage treatment plant discharges. Field testing the developed genotoxicity techniques indicated cytotoxicity and chromosome aberrations, but no DNA repair, in cultured fish and mammalian cells exposed to Sturgeon Bank sediment extracts. However, in a parallel test, an *in vivo* increase in micronuclei frequency in starry flounder could not be demonstrated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

Sincerest thanks are extended to the staff of the Environmental Carcinogenesis Unit at the B.C. Cancer Research Centre where this research was conducted. In particular, I would like to express my appreciation to my supervisors Dr. A. Acton and Dr. H. Stich, as well as Drs. Dunn, San, and Rosen for their help and encouragement. The technical assistance of Mrs. G. Wood, Mr. G. De Jong, Mr. G. Traxler (Dept. of Fisheries and Oceans, Nanaimo), and Dr. S. Campana (currently, Dept. of Fisheries and Oceans, Halifax) is gratefully acknowledged. Mrs. R. Pritchard and Mrs. A. Walton were instrumental in helping to produce the final thesis copy. Use of the B.C. Children's Hospital EEG Department word processor was kindly provided by Dr. P. Wong.

Finally, I would like to extend my deepest appreciation to my wife and family for their years of patience and invaluable help.

Financial support was provided by the B.C. Foundation for Non-Animal Research.

INTRODUCTION *

In a recent Perspectives article M. Gilbertson (1984) challenged the adequacy of approaching aquatic toxicology by focusing on laboratory experimentation. In his opinion, it would be much more informative to first undertake observations on fish populations to see whether any interesting anomalies are found, and then to form hypotheses which can be explored through laboratory experimentation and the development of further biochemical and histological methods.

Epidemiological studies of fish from local waters undertaken in the 1970's by Dr. Stich and co-workers at the B.C. Cancer Research Center determined that skin papillomas among flatfish populations occurred at elevated frequencies in association with contaminated environments (Stich et al., 1976, 1977a, 1977b). Collections of English sole (Parophrys vetulus) from Sturgeon Bank near Vancouver, for example, were found to have a 57% frequency of epidermal papillomas. Analyses of the Sturgeon Bank sediments, especially near the Iona Island sewage treatment plant outfall, showed high levels of the carcinogenic polycyclic aromatic hydrocarbon 3,4-benzopyrene (Dunn and Stich, 1976). However, further surveys failed to show an obvious correlation between the flatfish skin papilloma prevalences and 3,4-benzopyrene levels in bottom sediments which may be expected as the array of potentially carcinogenic chemicals will likely vary between different locations (Stich et al., unpublished results).

* see APPENDIX I - Publications from the Thesis Material

More recently it has been proposed that the flatfish epidermal papillomas or X-cell tumors are parasite infections (Alpers et al., 1977; Dawe, 1981) and not a result of exposure to chemical carcinogens (Stich et al., 1977a) and/or oncogenic viruses (Peters et al., 1978; Peters and Watermann, 1979). Further studies are required to better determine the etiology of these tumors.

Other epidemiological studies have correlated abnormally high tumor incidences in fish with chemically contaminated environments. Brown et al. (1973) found 4.4% of the fish surveyed from the Fox River in Illinois had tumors of the skin, stomach, and liver. They note that the river had elevated heavy metal, polycyclic aromatic hydrocarbon, and chlorinated hydrocarbon concentrations. Pierce et al. (1978) showed a 32% incidence of hepatomas in English sole (Parophrys vetulus) from the Duwamish River estuary near Seattle, Washington, and suspected chemical contaminants such as polychlorinated biphenyls as the etiological agent. In 1980, Black et al. reported dermal tumors in brown bullheads (Ictalurus nebulosus), gonadal tumors in goldfish x carp hybrids, and neurolemmomas in sheepshead (Aplodinotes grunniens) from an area contaminated with polycyclic aromatic hydrocarbons. Black et al. (1982) found elevated frequencies (up to 100%) of several tumor types in saugers (Stizostedion canadense) and walleye (Stizostedion vitreum) from Torch Lake, Michigan, which is heavily contaminated with copper-mining wastes. Also a recent Puget Sound study (Malins et al., 1984) associated chemical contaminants (aromatic hydrocarbons,

chlorinated organic compounds, and heavy metals) with up to 16% neoplasia incidence in English sole. A relationship is therefore apparent between fish inhabiting environmentally degraded areas and an elevated risk of neoplastic disease purportedly as a result of exposure to chemical contaminants.

Sampling and screening large numbers of fish for possible pathologies is a labour intensive, costly, and time-consuming project. Such epidemiological studies also provide only circumstantial evidence for an etiological agent. In order to better locate and delineate a contaminated area, and demonstrate probable carcinogenic activity through DNA-damaging events, a variety of relatively fast and inexpensive assays have been developed using bacterial or mammalian cells and enzyme preparations (Stich and San, 1981). In recent years fish biologists have adapted these assays, using fish in vivo or cultured fish cells and fish enzyme preparations to study the effects of chemical carcinogens, and to generate results which are more relevant to the aquatic ecosystem.

The U.B.C. Cancer Research Center laboratory was active in the development of the human fibroblast DNA repair assay in the early 1970's (Laishes, 1974; San, 1976) and later conducted surveys with the Chinese hamster ovary chromosome aberration test (Stich et al., 1977c; Lo and Stich, 1978; Stich et al., 1979). Since the target for many carcinogens is DNA (Heidelberger, 1975), these assays for DNA breakage and repair are of both practical and theoretical importance.

The research discussed here adapted such assays to be more relevant to the aquatic environment by employing fish

cells and activation enzymes, and undertook a comparative approach, contrasting fish cell responses to that of the mammalian cells normally used. In addition to studying model carcinogens such as N-methyl-N'-nitro-N-nitrosoguanidine and 4-nitroquinoline 1-oxide the effects of the carcinogenic mycotoxin aflatoxin B_1, to which rainbow trout are very sensitive (Sinnhuber et al., 1977), were also examined. Field applications of the assays related to our laboratory's earlier fish tumor work. Experiments focussed on the effects of, firstly, polycyclic aromatic hydrocarbons and, secondly, the Sturgeon Bank-Iona Island sewage treatment plant outfall area where there are elevated concentrations of polycyclic aromatic hydrocarbons, notably 3,4-benzopyrene.

MATERIALS AND METHODS

1. Chemicals *

Chemicals were obtained from the following sources: N-methyl-N'-nitro-N-nitrosoguanidine and 4-nitroquinoline 1-oxide from the Aldrich Chemical Company, Milwaukee, Wisconsin; aflatoxin B_1, 3,4-benzopyrene, 1,2-benzanthracene, and 1,2,5,6-dibenzanthracene from the Sigma Chemical Company, St. Louis, Missouri; pyrene from the Kodak Chemical Company, Rochester, New York; Aroclor 1254 from Analabs Incorporated, North Haven, Connecticut; [methyl-^{3}H]-thymidine (25 Ci/mmol specific activity) from Amersham Corporation, Amersham, England; and colchicine from the J.T. Baker Chemical Company, Phillipsburg, New Jersey. The N-acetoxy-2-acetylaminofluorene was a gift from the National Cancer Institute's Standard Chemical Carcinogen Repository in Chicago, Illinois, while the crude oil was provided by Shell Canada Ltd. Only N-methyl-N'-nitro-N-nitrosoguanidine dissolved directly into the culture media, all of the other test chemicals were prepared by initially dissolving in dimethylsulfoxide prior to dilution. Any other chemicals or solvents were of reagent or higher grade.

* see APPENDIX II - List of Abbreviations

2. Cell Cultures

a) Tissue culture cell lines

The human fibroblast cell line used was a stock culture originated from tissue explants at the Cancer Research Center. The chum salmon heart, rainbow trout ovary, rainbow trout

gonad, and fathead minnow were established cell lines obtained from Mr. G. Traxler (Pacific Biological Station, Nanaimo, British Columbia). The Chinese hamster ovary line was a stock lab culture which is available, as are the rainbow trout gonad and fathead minnow cell lines, from the American Type Culture Collection (Rockville, Maryland).

As there were no established central mudminnow (Umbra limi) cell lines these were initiated using the tissue explant technique (Wolf and Quimby, 1976; Wolf, 1979). Several live mudminnows were obtained from Mr. M. Rosenseld (Department of Zoology, University of British Columbia). The liver, spleen, fins, heart, swim bladder, and kidney were removed under sterile conditions and transferred to a saline solution containing antibiotics (83 ug/ml polymyxin B, 800 ug/ml bacitracin, 500 ug/ml neomycin), prior to plating in several 25cm^2 flasks (Falcon Plastics). After a 60 min attachment period, MEM (Eagle's minimal essential medium, Grand Island Biological Company) supplemented with 20% fetal calf serum was added and subsequently replaced at two week intervals. Though initially slow, their growth rate gradually increased and after 6-9 months fin (Ul-F) and heart (Ul-H) cell lines were firmly established.

b) Cell cultures

All cell lines were grown in 75cm^2 plastic flasks (Falcon Plastics) using MEM, antibiotics (streptomycin sulfate, 29.5 ug/ml; kanamycin, 100 ug/ml; fungizone, 2.5 ug/ml), and sodium bicarbonate (5 ml/800 ml medium for fish cells; 13.5 ml/800 ml medium for mammalian cells). Fish stock cultures

were maintained in sealed flasks at 18 C while mammalian stocks were grown at 37 C in a water-saturated CO_2 incubator. For experiments, cells were seeded onto coverslips (mammalian cells, $22mm^2$ coverslips in Falcon Plastic 3.5cm diameter plastic dishes; fish cells, 10mm x 35mm coverslips in stoppered Bellco Leighton tubes) and maintained in MEM supplemented with 10% serum.

c) DNA quantitation

The relative DNA content per cell in each of the cell lines was measured using the fluorescent stain propidium iodide and laser-based flow microfluorimetry (Krishan, 1975; Fried et al., 1976). Each cell culture was trypsinized to obtain a single-cell suspension which was fixed in 50% methanol then resuspended in ribonuclease (1 mg/ml in 0.2 M PBS, pH 7.0; Type III-A from bovine pancreas, Sigma Chemical Co., St. Louis, Missouri). This was replaced with a propidium iodide-sodium citrate solution (0.05 mg P.I./ml in 0.1% NaCit) and after a 20 min staining period the cells were resuspended in 0.2 M phosphate buffer (pH 7.0). Aliquots were drawn into a Coultar Epics V (Florida) flow microfluorimeter where the relative DNA content was measured and the data plotted by computer.

d) Preparation of S9

The S9 mixture was prepared as described by Ames et al. (1975). Fischer male rats (150-250 g; Charles River Breeding Laboratory, St. Constant, Quebec) and rainbow trout (150-250 g; Sun Valley Trout Farm, Mission, British Columbia) were injected intraperitoneally with Aroclor 1254 (500 mg/kg).

Rainbow trout were also injected (5 mg/kg) with an extract of crude oil (W. Penrose, Argonne National Laboratory, Argonne, Illinois, personal communication). The extract was prepared by vortexing 10 ml of crude oil with an equal volume of dimethylsulfoxide:water, which was removed and extracted with hexane. The hexane was volatilized in a rotary evaporator and the remaining tarry residue used for the injections. The livers were excised 5 days later, rinsed with cold 0.15 M KCl, pooled, and homogenized for 30-60 seconds in a polytron tissue homogenizer (Brinkman Instruments, Rexdale, Ontario) using 1g liver/3 ml of cold 0.15 M KCl. The homogenate was centrifuged in a refrigerated centrifuge (1-3 C) for 15 min at 9000 g. 5ml aliquots of the supernatant S9 fraction were pipetted into polypropylene tubes (Falcon Plastics) and stored at -80 C.

The S9 mixture for the DNA repair experiments contained 200 ul/dish of S9 and the enzyme cofactors NADP (1.8 mg/dish), G6P (2.7 mg/dish), and NADPH (0.6 mg/dish) dissolved in 0.3 ml PBS. For the chromosome aberration experiments the S9 mixture contained 50 ul/dish of S9 and the cofactors NADP (1.4 mg/dish) and G6P (0.7 mg/dish) initially dissolved in Hepes buffer.

e) DNA repair synthesis

For each experiment, approximately 20,000 HF cells (passage 5-7), 60,000 CHO cells (continuously cultured), and 80,000 cells for each fish cell line (RTG, passage 70-74; RTO, passage 15-18; CH, passage 19-22; FHM, passage 88-91; Ul-H, passage 9; Ul-F, passage 11) were seeded onto coverslips and kept in MEM supplemented with 10% serum until the cells

were about 50% confluent. Cell division was inhibited by arginine-deprivation in order to prevent false positives resulting from the incorporation of ^{3}HTdR into DNA during replication. The cells were transferred into arginine-deficient medium (ADM) supplemented with 2.5% serum and incubated for a further 4-5 days.

ADM with 2.5% serum was used to dilute the test chemicals. ^{3}HTdR was also diluted with ADM to attain a working concentration of 10 uCi/ml. In an experiment the culture medium was removed and 1.0 ml of ^{3}HTdR and test chemical added for the treatment period. Where enzyme activation was required, a 0.5 ml aliquot of S9 mixture (S9 plus cofactors) was added to 0.5 ml of the test chemical and 1.0 ml ^{3}HTdR.

Upon completion of the chemical treatment, the cells were treated with sodium citrate or potassium chloride (mammalian cells, 1% NaCit; Ul-H and Ul-F, 0.4% KCl; all other fish cell lines, 0.5% NaCit) for 10 min, immediately followed by fixation in ethanol/acetic acid (3:1) for 10-20 min. Air-dried coverslips were mounted on slides using paraffin and autoradiographically processed using an 18 or 30 day exposure to Kodak Nuclear Track Emulsion (NTB-3; Eastman Kodak, Rochester, New York). After developing the cells were stained with 2% aceto-orcein (50:50, acetic acid/water) and a second coverslip mounted with Permount. DNA repair synthesis was measured as the mean number of silver grains over nuclei. The background grain count, which varied from approximately 0 to 5 grains, was subtracted from the nuclear grain count. At least

30 nuclei were scored for each data point which were plotted with a standard deviation on the mean.

f) Chromosome aberrations

150,000 Ul-H, 40,000 CHO, or 80,000 HF cells were seeded per coverslip and grown to 60-80% confluency. For test chemical treatments the culture medium was removed and 1.0 ml of chemical in MEM with 2.5% serum added. Enzyme activation was provided as required by adding 0.5 ml of an S9 mixture (S9 plus cofactors) to 0.5 ml of the test chemical. Following a 3h treatment period the cells were rinsed with MEM then incubated in 1.5 ml MEM with 10% serum (20% for Ul-H) for 20h (Ul-H, CHO) or 28h (HF). 4h prior to sampling 0.1 ml of colchicine was added. The coverslips with cells were then processed and fixed as noted in the section on DNA repair methods. Air-dried coverslips were stained with 2% aceto-orcein (50:50 acetic acid : water), dehydrated, and mounted on slides with Permount. In general, 100 metaphase plates per coverslip were analyzed for chromosome breaks and exchanges, the most common forms of aberrations observed in chromosome preparations from these cell lines.

g) Micronuclei

120,000 Ul-H, 120,000 Ul-F, 14,000 CHO, or 30,000 HF cells were seeded onto coverslips and grown to 20-30% confluency in MEM with 10% serum. The coverslips of cells were then treated with a test chemical in MEM with 2.5% serum for 3h following the chromosome aberration assay methodology. After the treatment period the chemical was rinsed from the coverslips with MEM and the cells were incubated in MEM with

10% serum until sampled at 16, 48, 80, 112, and 144h. Coverslips were processed and fixed as noted in the section on DNA repair methods. Air-dried coverslips were stained with aceto-orcein, dehydrated, and mounted on slides. In general, for each data point 1000 cells were examined for the presence of micronuclei.

3. Primary Cells

a) Isolation of the cells

Liver, stomach, and intestine were removed from 150- to 200- g rainbow trout. Using a syringe, the stomach and intestine were flushed with cool, sterile balanced salt solution (BSS) supplemented with antibiotics (polymyxin B, 83 mg/l; bacitracin, 800 mg/l; neomycin, 5 mg/l). The liver was rinsed with BSS, then minced with 5 ml BSS in a sterile plastic dish (Falcon Plastics). The stomach and intestine were cut longitudinally to expose the mucosa. Each tissue was placed into a sealed sterile polycarbonate Erlenmeyer flask containing 10 ml BSS with 0.5% pronase, and incubated at 20 C for 45 to 60 min with occasional agitation to loosen the cells. Following the incubation period, the solution was pipetted off and the remaining tissue washed with BSS. The enzyme solution and washings were pooled and passed through a piece of sterile gauze to remove large tissue pieces. The cell suspensions were centrifuged at 600 rpm for 5 min, resuspended in 6 ml ADM with 2.5% serum, and then centrifuged and resuspended in fresh ADM with 2.5% serum. Cell viability was tested at 0 and 6 hours using the trypan blue exclusion technique.

b) DNA repair synthesis with primary cells

ADM with 2.5% serum and ^{3}HTdR (final working concentration 10 uCi/ml) was used for making the test chemical dilutions. 1 ml aliquots of cell suspension and chemical:^{3}HTdR were added to 3.5 cm plastic dishes (Falcon Plastics), which were placed in a sealed CO_2-flushed (5% CO_2 in air) container at 18-20 C for 6h.

At the end of the treatment period the cells were pipetted into test tubes, centrifuged, and resuspended in ethanol:acetic acid (3:1). After 10 min the cells were again centrifuged and all but approximately 0.2 ml of the fixative removed. The remaining fixative was used to resuspend the cells, which were dropped onto microscope slides. Air-dried slides were coated with Kodak Nuclear Track Emulsion (NTB-3) and kept in desiccated light-tight boxes at 4 C for 30 days. After developing, the cells were stained with 2% aceto-orcein and the amount of DNA repair determined. The background grain count was estimated from an equivalant area adjacent to each nucleus and subtracted from its count. DNA repair synthesis was measured as the mean number of silver grains over at least 30 nuclei for each data point. These were plotted with a standard deviation on the mean.

4. Tissue Slices

DNA repair synthesis in tissue slices

Starry flounder (Platichthys stellatus) with epidermal papillomas were collected by beach seine from Bellingham Bay (December, 1980) and kept in an aquarium at the Cancer

Research Center. Fish selected for the experiments were killed by concussion and their papillomas removed. Slices of the papillomas were placed in 3.5 cm plastic dishes (Falcon Plastics) with 1.0 ml of test chemical in 2.5% ADM and ^{3}HTdR (10 uCi/ml). The dishes were placed in a sealed CO_2-flushed (5% CO_2 in air) container at 18-20 C for a 6h treatment period. The tissue slices were then removed, fixed in formalin, and histologically processed. 2, 5, and 10 micron sections mounted on slides were coated with Kodak Nuclear Track Emulsion (NTB-3) and held in light-tight boxes at 4 C for 30 days. After developing, the tissue sections were stained using hematoxylin and eosin, and the cells examined for evidence of DNA repair.

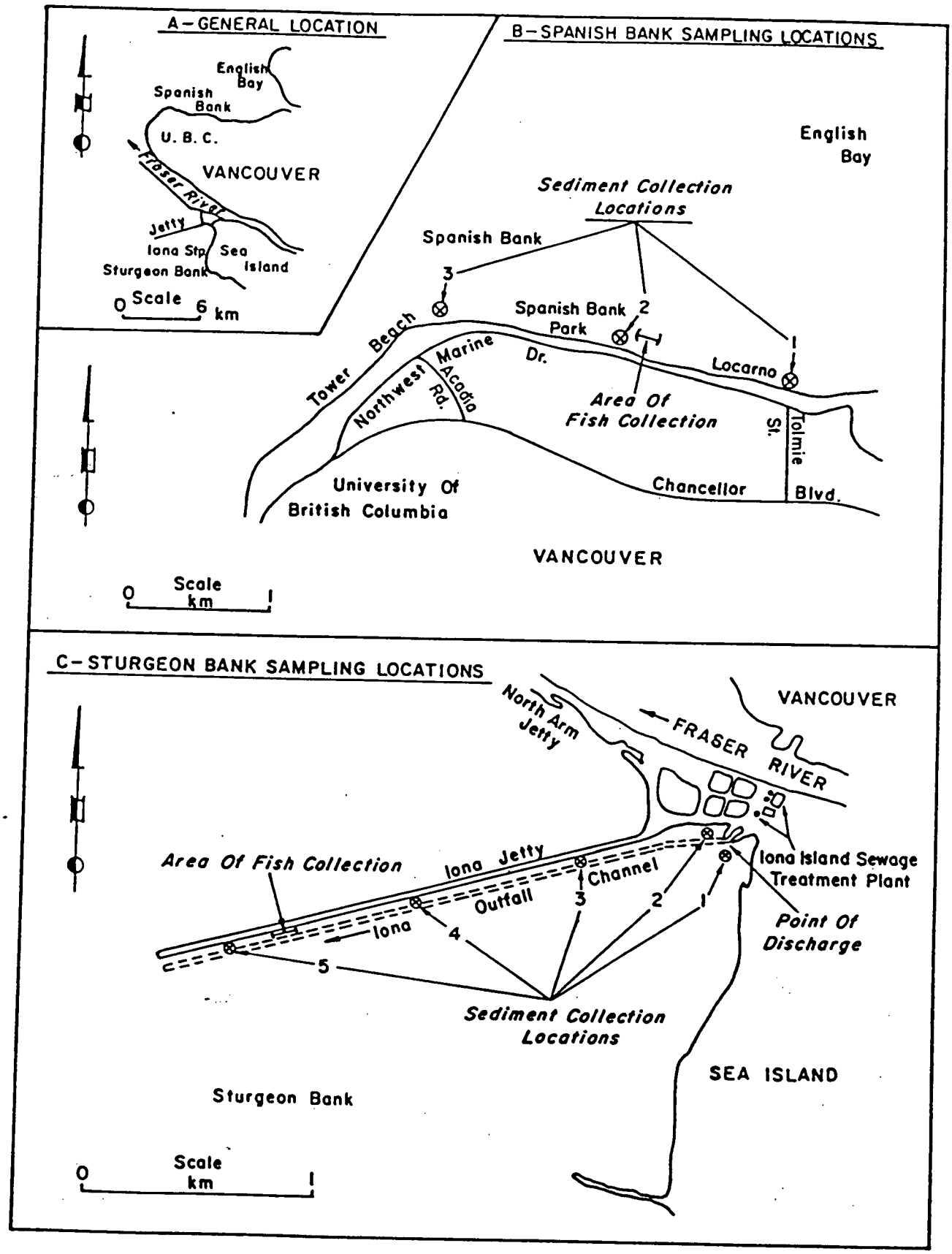
5. Sediments

Collection, preparation, and assaying of sediment samples

Sediment samples were collected from the Sturgeon Bank-Iona Island jetty area on October 31, 1983, and the Spanish Bank area on November 20, 1983 (Figure 1). At each of the 5 Sturgeon Bank and 3 Spanish Bank sampling locations the top 5 cm of sediment from three 30 cm^2 areas was removed and placed in plastic bags. The sediment samples were frozen at -20 C until processed. After thawing the sediments for a particular location, approximately equal portions of the location's three sediment samples were combined and freeze-dried. 500 g of a freeze-dried uniform sample was then placed in a clean glass bottle, and extracted three times with 500 ml portions of methylene chloride (HPLC grade, Fisher Chemicals). Suspended

FIGURE 1

Location of the fish and sediment collection sites at Sturgeon and Spanish Banks (Greater Vancouver Sewerage and Drainage District, 1983).


A – GENERAL LOCATION
English Bay
Spanish Bank
U. B. C.
VANCOUVER
Fraser River
Jetty
Iona Stp
Sea Island
Sturgeon Bank
0 Scale 6 km
B – SPANISH BANK SAMPLING LOCATIONS
English Bay
Sediment Collection Locations
Spanish Bank
3
Spanish Bank Park
2
1
Tower Beach
Marine Dr.
Northwest Acadia Rd.
Locarno
Area Of Fish Collection
St.
Tolmie
University Of British Columbia
Chancellor Blvd.
VANCOUVER
0 Scale km 1
C – STURGEON BANK SAMPLING LOCATIONS
North Arm Jetty
VANCOUVER
FRASER RIVER
Area Of Fish Collection
Iona Jetty
Channel
Outfall
Iona
4
3
2
1
5
Iona Island Sewage Treatment Plant
Point Of Discharge
Sediment Collection Locations
SEA ISLAND
Sturgeon Bank
0 Scale km 1

particles were removed from the combined supernatants by settling and filtration (Whatman #1 filter). The methylene chloride was removed with a rotary evaporator and the residue dissolved in dimethylsulfoxide. The extracts were stored at -80 C until tested for genotoxic activity in the DNA repair and chromosome aberration assays, following the previously discussed methodology.

6. Flatfish

Collection of flatfish and examination for micronuclei

Fish were collected by beach seine from Sturgeon Bank on October 30, 1983, and Spanish Bank on November 19, 1983 (Figure 1). Starry flounder (Platichthys stellatus), the predominant species found, were transported to the laboratory and held in aquaria. Within 72h following their collection, 25 fish from Sturgeon Bank (length, 16.5 to 28 cm) and 22 from Spanish Bank (length, 15.5 to 25 cm) were killed and cell samples from the mouth, liver, and blood obtained. The bottom of the mouth was cut back to the operculum to expose the buccal epithelium. By drawing a scalpel blade across the epithelial surface, cells were removed which were then spread on slides. Cells were isolated from the liver following the procedure described in the section on the isolation of primary cells methods. Blood samples were obtained by removing the tail and collecting drops of blood on a slide. Liver and blood cell suspensions dropped onto slides were drawn into a fairly uniform cell layer by passing a second slide across its surface. Air-dried slides were stained by the periodic acid

Schiff method and counterstained with fast green. A coverslip was attached with Permount. 500-1000 buccal epithelial, liver, or red blood cells were examined for micronuclei.

RESULTS

A. DNA Repair Synthesis

1. Repair in Cultured Cells

a) Cell line comparison

Significant levels of DNA repair were observed following exposure of all mammalian and fish cell lines to MNNG, 4NQO, NA2AAF, and rat S9 activated AFB_1 (Figure 2). Dose-dependent responses were observed with all exposures, the level of response varying in the order HF > CHO > all the fish cell lines. DNA repair synthesis was observed to peak in all of the cell lines at approximately the same concentration of a particular chemical, and the repair magnitude was dependent on the test chemical used. Closer examination of the fish cell dose-response curves (Figure 3) indicates relatively little variation between lines with regard to sensitivity or maximal response. Only nominal DNA repair activity was detected following exposure to non-activated AFB_1 (Figure 4).

b) Investigation into the low fish cell DNA repair response

A number of factors which could account for the apparent low fish cell DNA repair response were examined.

To ensure that grain count differences were not solely due to possible poikilothermic : homeothermic membrane differences resulting in variation in test chemical uptake, the amount of repair following irradiation with ultraviolet light was measured (Table 1). These data confirmed the test chemical data which showed the magnitude of the DNA repair response varied HF > CHO > RTG.

FIGURE 2

DNA repair synthesis measured in cultured HF (□), CHO (▽), RTG (●), RTO (▼), FHM (■), and CH (▲) cells exposed to (A) MNNG, (B) 4NQO, (C) NA2AAF, and (D) rat S9 activated AFB_1. Assay conditions: 37 C (mammalian cells) and 18 C (fish cells), 3h test chemical treatment period, emulsion-coated slides kept in light-tight boxes for 18 days prior to developing. Mean grain counts plotted with standard deviations.

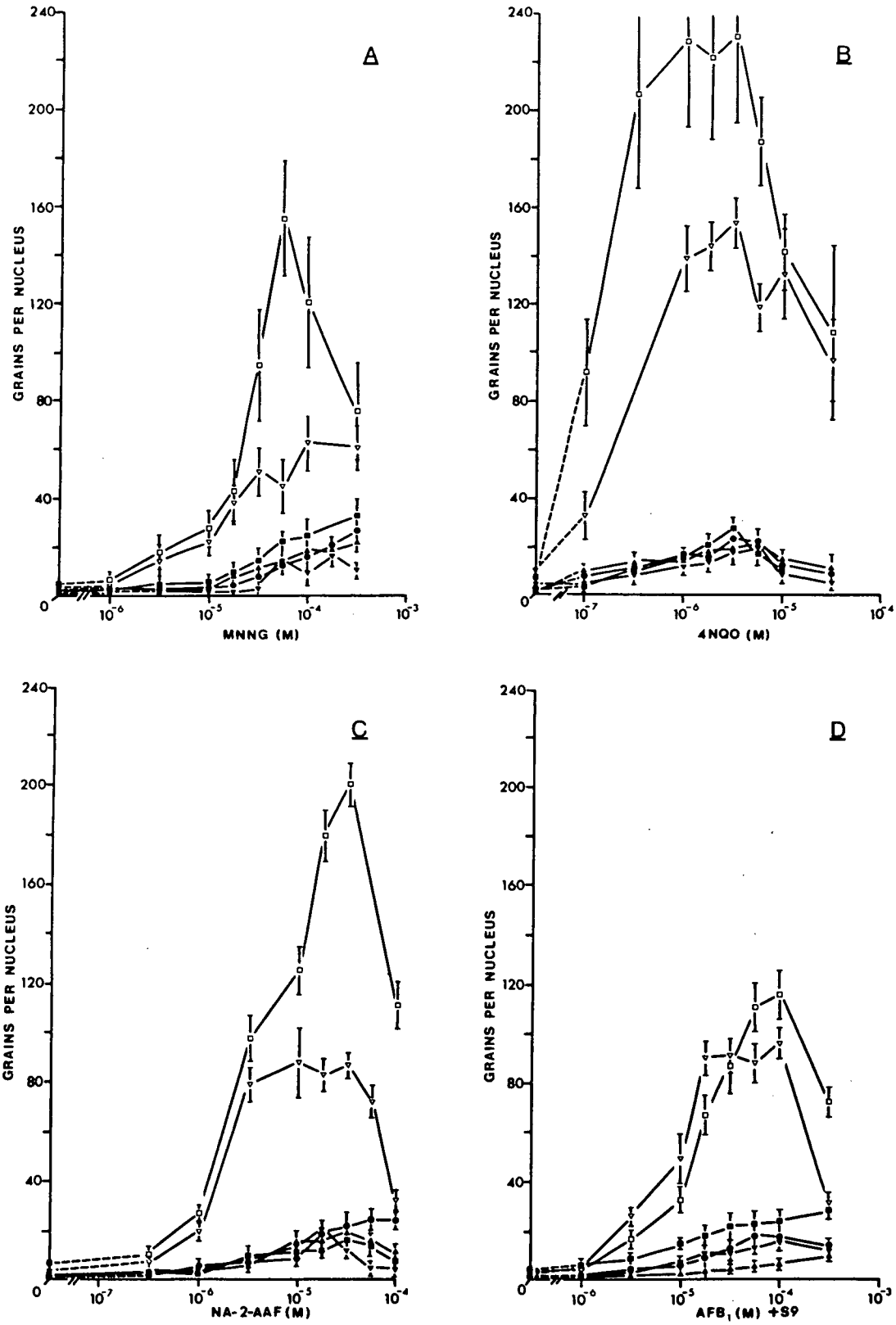
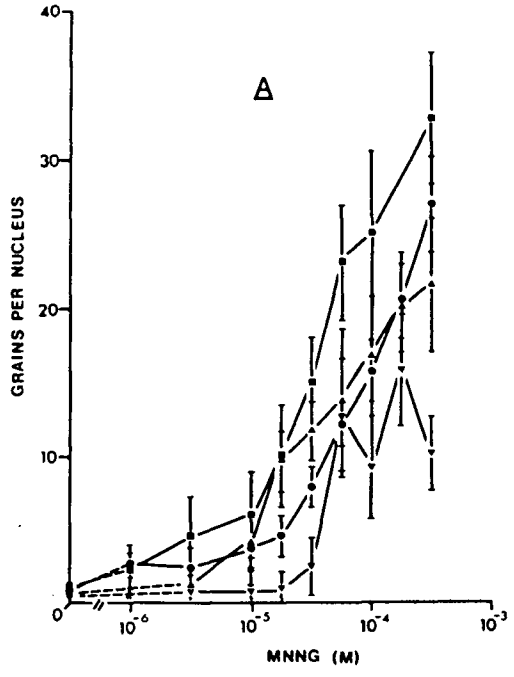
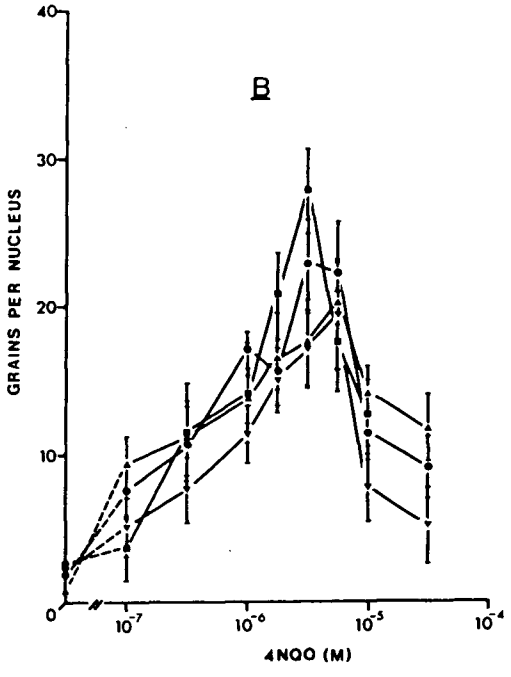
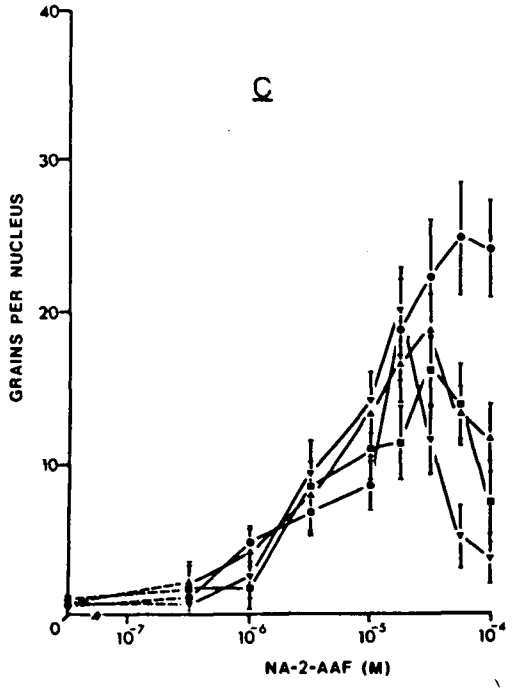

A
B
C
D
GRAINS PER NUCLEUS
MNNG (M)
4NQO (M)
NA-2-AAF (M)
AFB1 (M) +S9

FIGURE 3

The data from FIGURE 2 for the DNA repair synthesis in cultured RTG (●), RTO (▼), FHM (■), and CH (▲) cells exposed to (A) MNNG, (B) 4NQO, (C) NA2AAF, and (D) AFB, replotted to examine the difference in repair magnitude between the fish cell lines.


A
GRAINS PER NUCLEUS
MNNG (M)


B
GRAINS PER NUCLEUS
4NQO (M)


C
GRAINS PER NUCLEUS
NA-2-AAF (M)

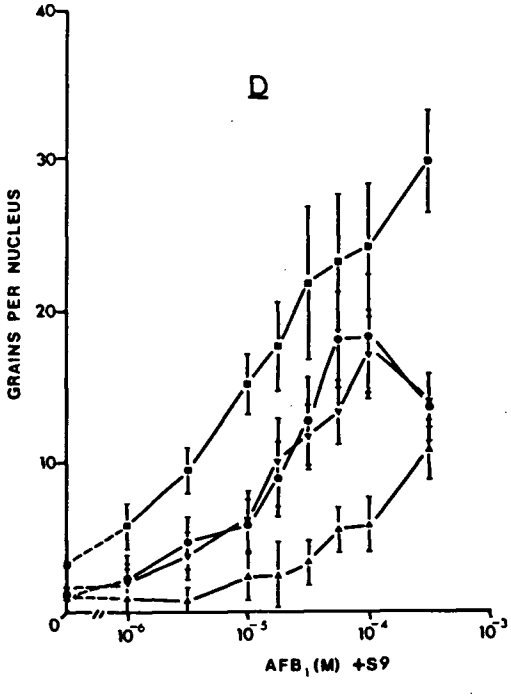

D
GRAINS PER NUCLEUS
AFB1 (M) +S9

FIGURE 4

DNA repair synthesis measured in cultured HF (□), CHO (▽), RTG (●), RTO (▼), FHM (■), and CH (▲) cells exposed to non-activated AFB_1. Assay conditions: 18 C (fish cells) and 37 C (mammalian cells), 3h test chemical treatment, emulsion-coated slides kept in light-tight boxes for 18 days prior to developing. Mean grain counts plotted with the standard deviation on the mean.

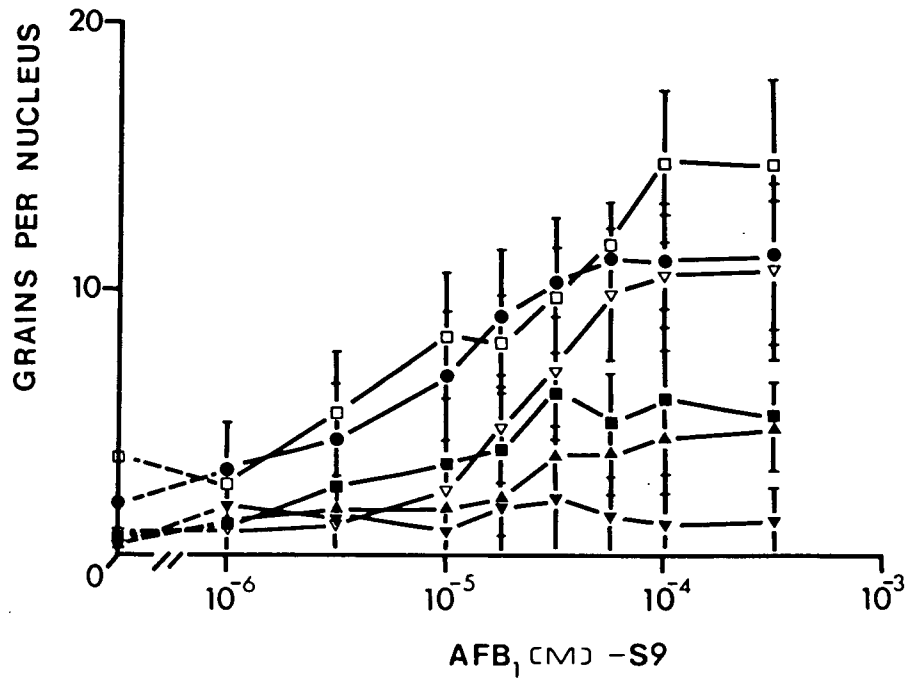

GRAINS PER NUCLEUS
20
10
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
AFB_1 (M) −S9

TABLE 1

DNA repair synthesis in HF, CHO, and RTG cells exposed to ultraviolet light. Assay conditions: irradiated coverslips incubated at 18 C for 3 h, emulsion-coated slides kept in light-tight boxes for 18 days prior to developing.

UV Dose ($ergs/mm^2$)	Grains per Nucleus ± Standard Deviation		
	RTG	CHO	HF
80	38 ± 7	Saturated	Saturated
60	29 ± 6	86 ± 7	Saturated
40	22 ± 3	58 ± 8	192 ± 18
20	17 ± 3	23 ± 4	147 ± 12
0	2 ± 2	2 ± 2	2 ± 2

As the low amount of fish cell DNA repair could be explained by a delayed or prolonged repair response, the duration of repair following test chemical treatment was investigated. Similar time course responses were found after treating HF, CHO, and RTG cells with MNNG or 4NQO (Figure 5). The level of repair peaked at approximately 3h, then fell off rapidly, attaining low, essentially background levels by 16-20h.

The cell line variation in repair magnitude could simply be the result of the amount of DNA "target". However, the relative DNA quantity per cell indicated only 10 to 15% less DNA in the fish than in the mammalian cell lines.

c) Increasing the fish cell DNA repair response

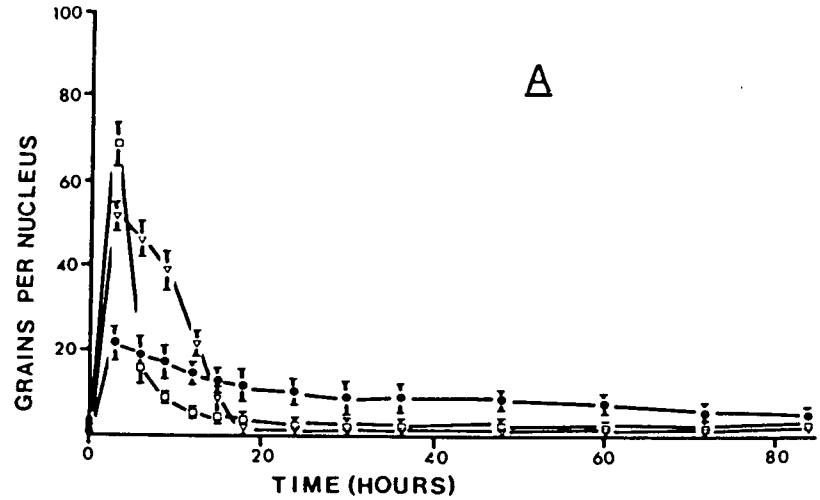
It was decided that only if a fish cell repair response in excess of 50 grains per nucleus could be achieved, would the assay be deemed sufficiently sensitive to be worth pursuing. Several factors were considered which could potentially improve the repair response.

Extending the cells' incubation period in ^{3}HTdR (Figure 6) to 6h following MNNG treatment resulted in higher grain counts. Presumably a longer incubation period permits more DNA to be repaired and hence a greater incorporation of ^{3}HTdR. No toxic effects were observed with the longer ^{3}HTdR incubation time. Grain count increases are not proportional to increases in ^{3}HTdR incubation time. This is likely a consequence of the rapidly falling level of repair following test chemical treatment (Figure 5).

Assay temperature was found to influence grain production

FIGURE 5

Duration of DNA repair synthesis in cultured HF (□), CHO (▽), and RTG (●) cells exposed to (A) MNNG or (B) 4NQO. The cells sampled at 3h were simultaneously treated with the test chemical and ^{3}HTdR for the initial 3h segment, while the rest of the cells were treated with only the chemical for this initial period, then later pulsed with ^{3}HTdR for 3h. Assay conditions: 37 C (mammalian cells) and 18 C (fish cells), emulsion-coated slides kept in light-tight boxes for 18 days prior to developing. Mean grain counts plotted with a standard deviation on the mean.


A
GRAINS PER NUCLEUS
100
80
60
40
20
0
20
40
60
80
TIME (HOURS)

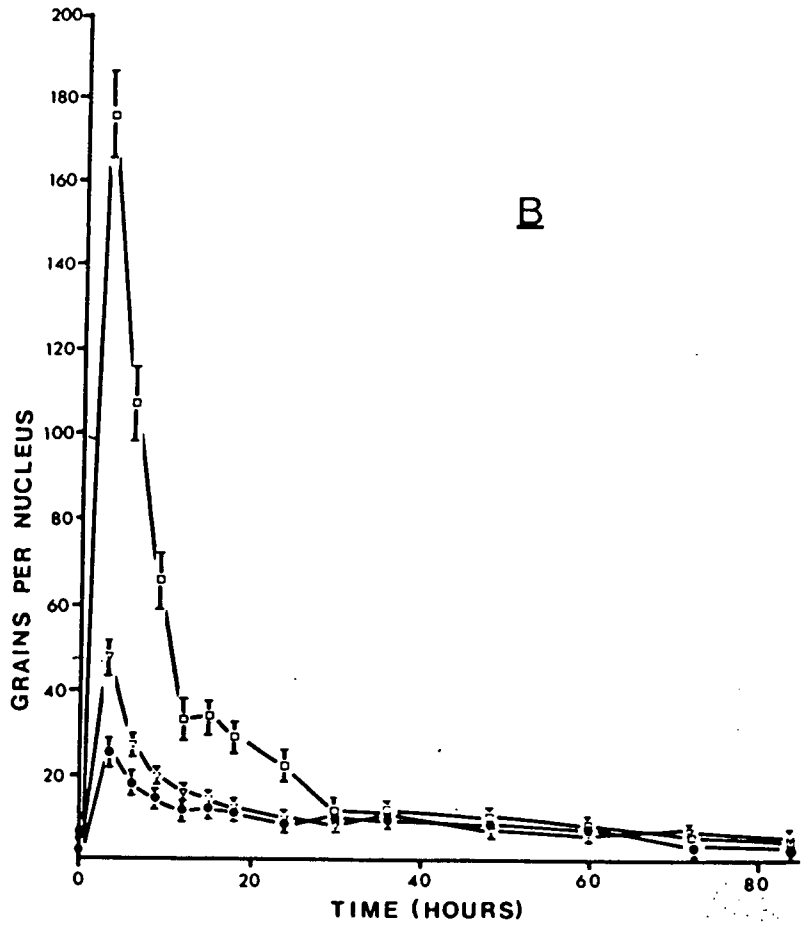
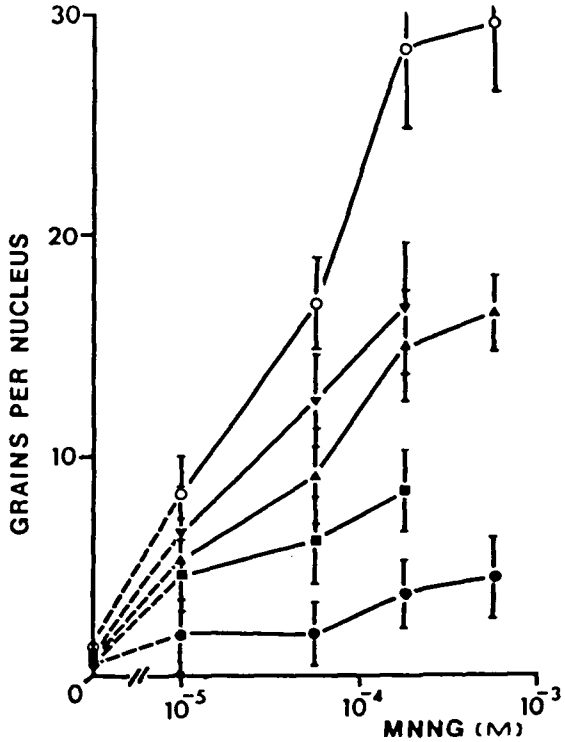

B
GRAINS PER NUCLEUS
200
180
160
140
120
100
80
60
40
20
0
20
40
60
80
TIME (HOURS)

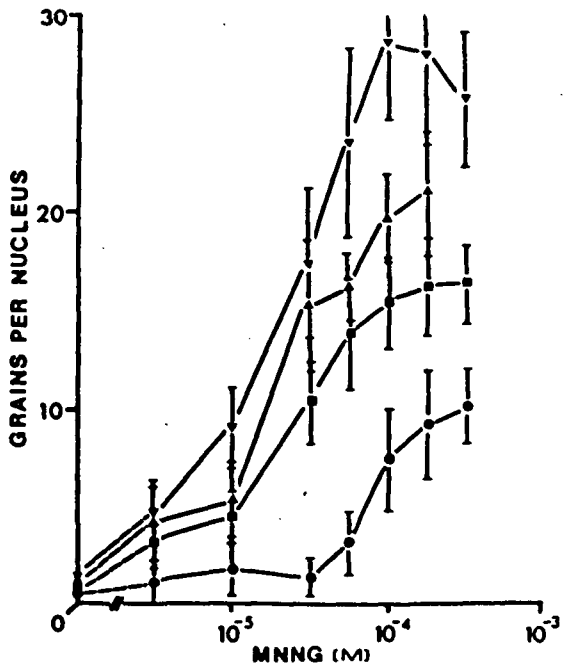
FIGURE 6

Effect of increasing ^{3}HTdR treatment time (● , 30 min; ■ , 60 min; ▲ , 90 min; ▼ , 100 min; O , 360 min) on DNA repair in cultured RTG cells following a 30 min MNNG treatment. Assay conditions: 18 C, emulsion-coated slides kept in light-tight boxes for 18 days prior to development. Mean grain counts plotted with a standard deviation on the mean.

FIGURE 7

RTG cells exposed to emulsion for varying periods of time up to 28 days (7 days,●; 14 days,■; 21 days,▲; 28 days,▼) prior to developing. Assay at 18 C. Mean grain counts plotted with a standard deviation on the mean.


30
20
10
0
GRAINS PER NUCLEUS
10^{-5}
10^{-4}
10^{-3}
MNNG (M)


30
20
10
0
GRAINS PER NUCLEUS
10^{-5}
10^{-4}
10^{-3}
MNNG (M)

(Figure 8). Conducting the assay over a temperature range from 4 to 25 C indicated maximum repair synthesis at 25 C. Cell death from the temperature change was not observed during the experiment. A number of karyorrhexic nuclei were observed in RTG cells maintained for several passages at 25 C, possibly indicating that the stability of their DNA may be influenced by temperature.

Increasing the duration of exposure to the emulsion also increased nuclear grain counts (Figure 7). A longer emulsion exposure period permits more ^{3}HTdR to decay and react with the emulsion to produce more grains over the nucleus. As the half-life of ^{3}HTdR is approximately 12 years (Rogers, 1979) the rate of ^{3}HTdR decay should be constant over the 30 day exposure period used here. The results, as expected, show a proportional grain count increase with increases in emulsion exposure time.

Increased grain production was found when the 4NQO treatment was lengthened to approximately 30 min (Figure 9). Only a slight grain count increase is realized between 30 to 60 min of chemical treatment and the slight decrease beyond 60 min may indicate a toxic effect.

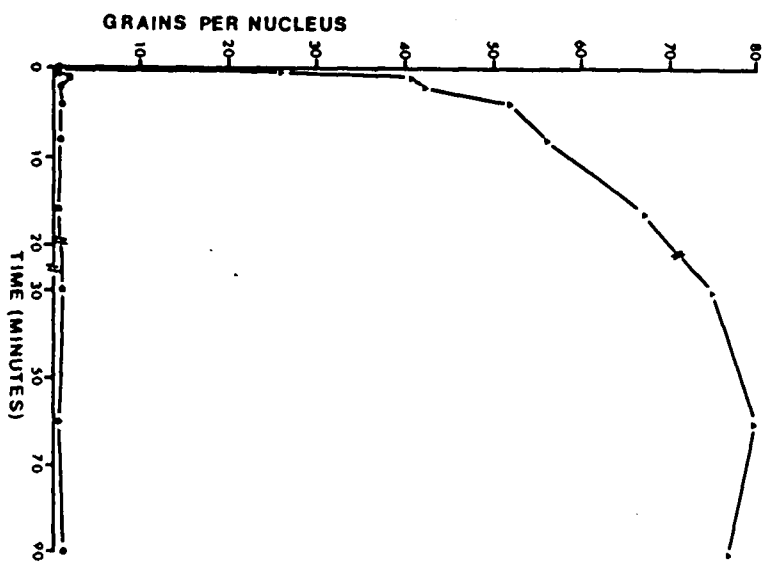
Other factors were investigated, none of which enhanced grain production in the RTG cells. It was not known if the nutritional requirements of RTG cells, with possible subsequent effects on DNA repair capability, would be impaired by prolonged cell maintenance in ADM prior to the assay, or the 7.5% lower serum concentration in the ADM. However, varying the cell division arrest time from 1 to 7 days in 2.5%

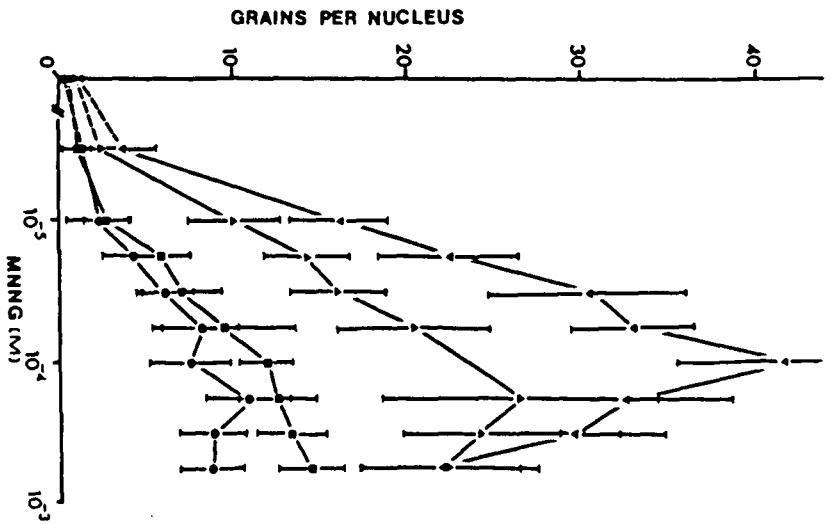
FIGURE 8

Effect of assay temperature (4 C, ● ; 11 C, ■ ; 18 C, ▲ ; 25 C, ▼) on DNA repair in cultured RTG cells treated simultaneously with ^{3}HTdR and MNNG. Assay conditions: cells seeded at 18 C with a 48h acclimatization period prior to the experiment, emulsion-coated slides kept in light-tight boxes for 18 days prior to development. Mean grain counts plotted with a standard deviation.

FIGURE 9

Increase in DNA repair in RTG cells at 25 C with increased treatment time in $5x10^{-4}$M 4NQO (▲,experimental; ●,control). 4NQO treatment was followed by a 6h ^{3}HTdR treatment, and a 30 day emulsion exposure. Mean grain counts are plotted.


GRAINS PER NUCLEUS
0
10
20
30
40
50
60
70
80
TIME (MINUTES)
0
10
20
30
50
70
90


GRAINS PER NUCLEUS
0
10
20
30
40
MNNG (M)
10^{-5}
10^{-4}
10^{-3}

ADM (Figure 10) prior to MNNG exposure had no effect on the level of RTG DNA repair, while varying the ADM serum concentration from 0 to 10% resulted in a slight lowering of grain counts (Figure 11). Another factor which could have limited RTG cell repair was lack of ^{3}HTdR uptake. But the 10 uCi/ml of ^{3}HTdR appears not to be limiting as grain production was not enhanced beyond 2.5 uCi/ml (Figure 12). That ^{3}HTdR is adequately penetrating the cells is further evidenced by a few heavily grained (ie. in S-phase) nuclei observed in control preparations. Lastly RTG cell cultures differing by over 30 passages, when treated with MNNG and ^{3}HTdR, failed to demonstrate a loss of repair capability with time in culture (Figure 13).

By conducting the DNA repair assay at 25 C instead of 18 C, increasing the ^{3}HTdR treatment period from 3 to 6 h, and extending the emulsion exposure period from 18 to 30 days, RTG cell grain counts were increased 2 to 3-fold and mean counts in excess of 50 grains were achieved (Figure 14). Background grain counts were also observed to increase slightly but the effect was minimized by using a low cell density on each coverslip, and by thorough rinsing of the coverslip during the fixation procedure, to remove non-incorporated ^{3}HTdR.

d) Effect of DMSO on DNA repair synthesis

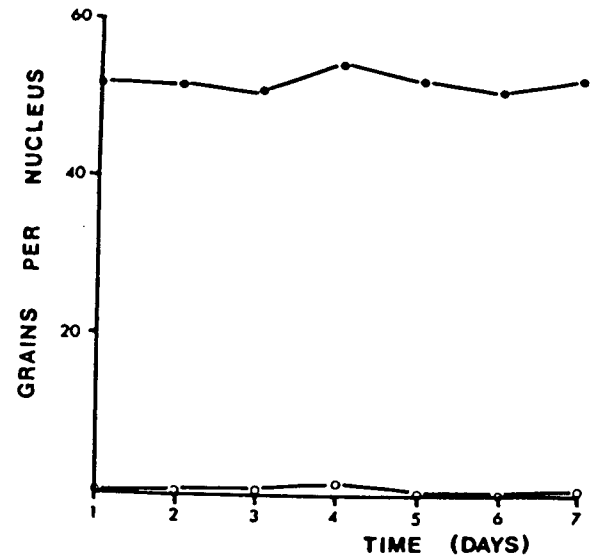
As DMSO was used for initial dissolving of test chemicals its effect on DNA repair was examined (Figure 15). Ultraviolet light irradiated RTG cells exhibited less repair at final DMSO concentrations in excess of 1%. Cell loss from coverslips was observed at 5% and 10%, indicating that the

FIGURE 10

DNA repair in cultured RTG cells kept in 2.5% ADM (● , experimental; O , control) for a period varying from 1 to 7 days prior to a 6h simultaneous exposure to $5x10^{-4}$M MNNG and ^{3}HTdR. Assay conditions: 25 C, emulsion-coated slides kept in light-tight boxes for 30 days prior to developing. Mean grain counts are plotted.

FIGURE 11

DNA repair in cultured RTG cells given a 3h exposure to ^{3}HTdR and MNNG diluted with ADM supplemented with varying concentrations of fetal calf serum (▼ , 0% serum; ■, 5% serum; ▲ , 7.5% serum; ● , 10% serum). Assay conditions: 18 C, emulsion-coated slides kept in light-tight boxes for 18 days. Mean grain counts are plotted.


GRAINS PER NUCLEUS
60
40
20
1
2
3
4
5
6
7
TIME (DAYS)

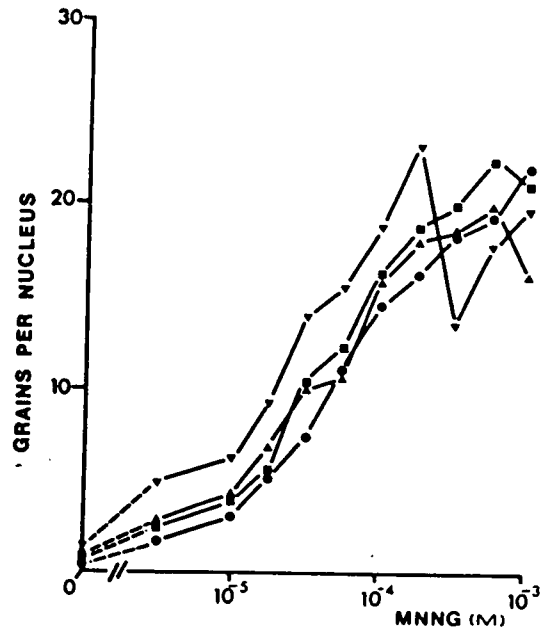
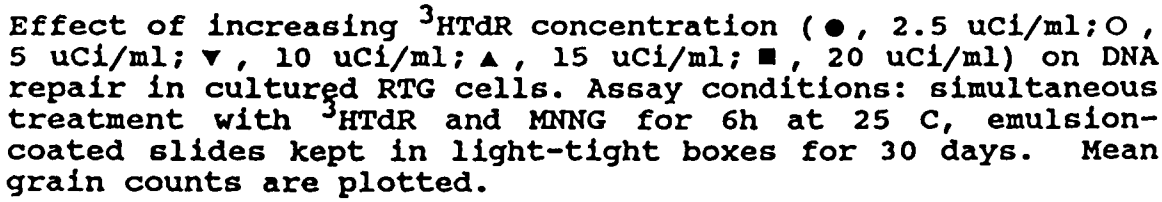
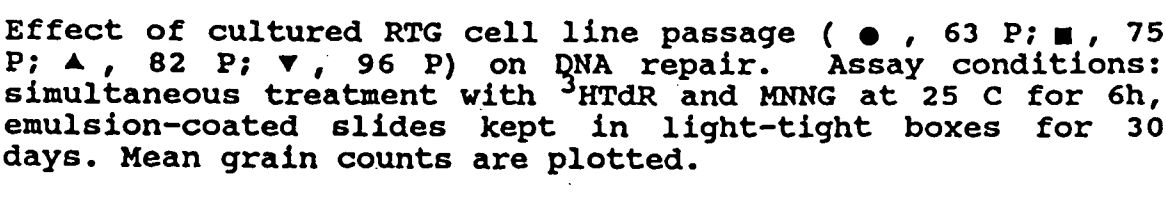

GRAINS PER NUCLEUS
30
20
10
0
10^{-5}
10^{-4}
10^{-3}
MNNG (M)

FIGURE 12

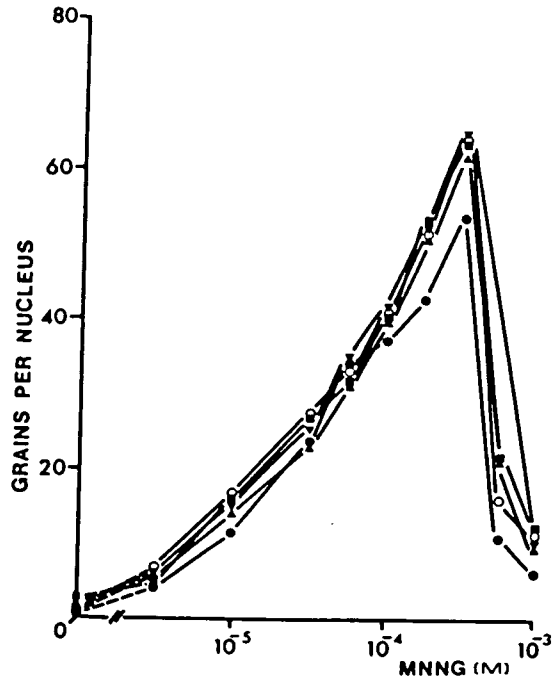


Effect of increasing ^{3}HTdR concentration (● , 2.5 uCi/ml; O , 5 uCi/ml; ▼ , 10 uCi/ml; ▲ , 15 uCi/ml; ■ , 20 uCi/ml) on DNA repair in cultured RTG cells. Assay conditions: simultaneous treatment with ^{3}HTdR and MNNG for 6h at 25 C, emulsion-coated slides kept in light-tight boxes for 30 days. Mean grain counts are plotted.

FIGURE 13



Effect of cultured RTG cell line passage (● , 63 P; ■ , 75 P; ▲ , 82 P; ▼ , 96 P) on DNA repair. Assay conditions: simultaneous treatment with ^{3}HTdR and MNNG at 25 C for 6h, emulsion-coated slides kept in light-tight boxes for 30 days. Mean grain counts are plotted.


80
60
40
20
0
GRAINS PER NUCLEUS
10^{-5}
10^{-4}
10^{-3}
MNNG (M)

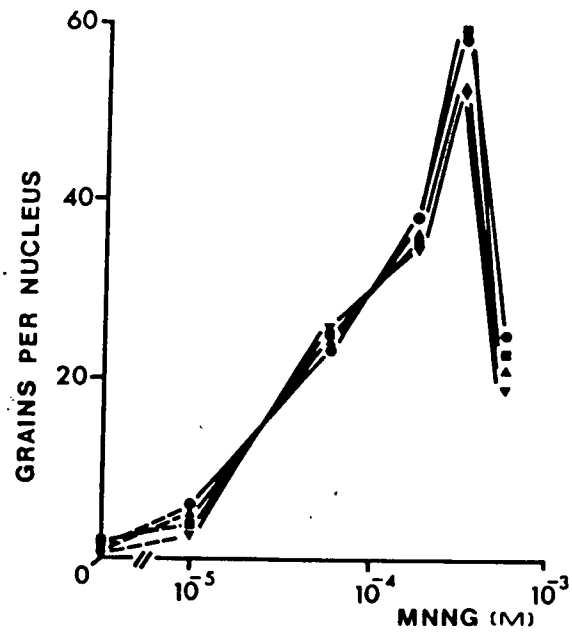

60
40
20
0
GRAINS PER NUCLEUS
10^{-5}
10^{-4}
10^{-3}
MNNG (M)

FIGURE 14

Comparison of DNA repair in cultured RTG cells exposed to MNNG (●) or 4NQO (■) utilizing (A) the original experimental conditions (18 C assay temperature, 3h ^{3}HTdR and test chemical treatment, and 18 day emulsion exposure period) and (B) the conditions found to enhance grain production (25 C assay temperature, 6h ^{3}HTdR and test chemical treatment, and 30 day emulsion exposure period). Mean grain counts plotted with a standard deviation on the mean.

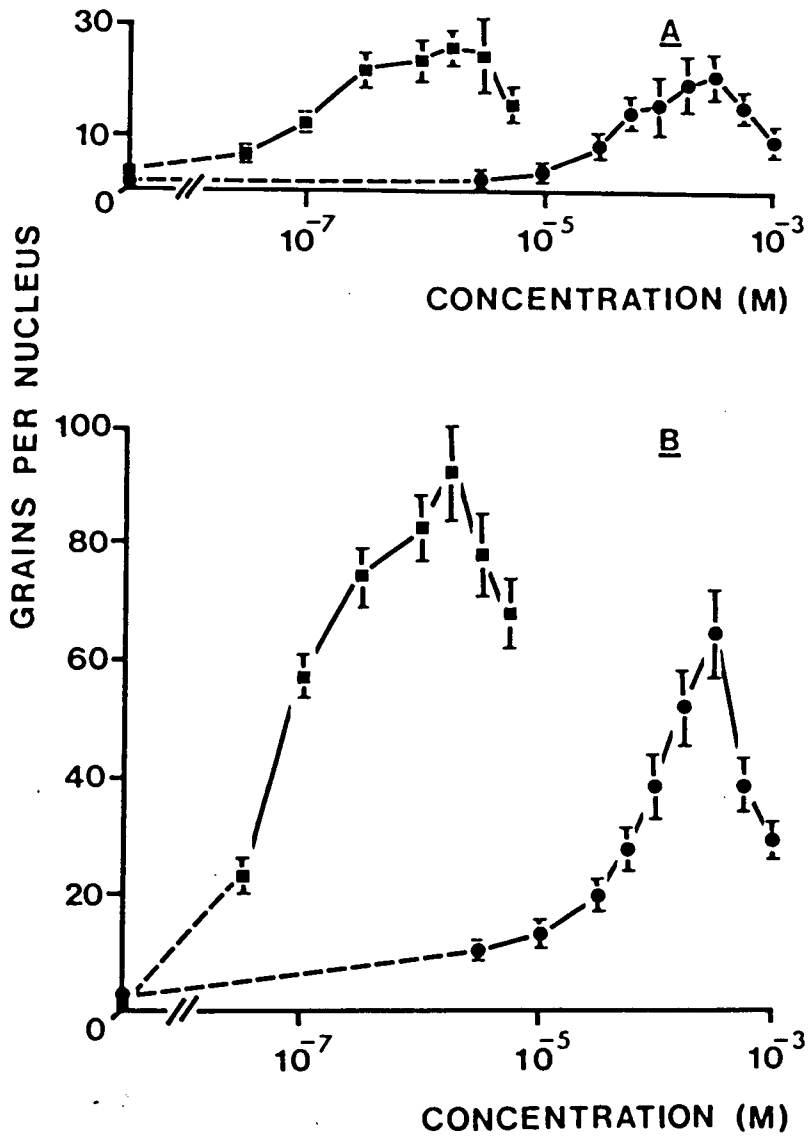
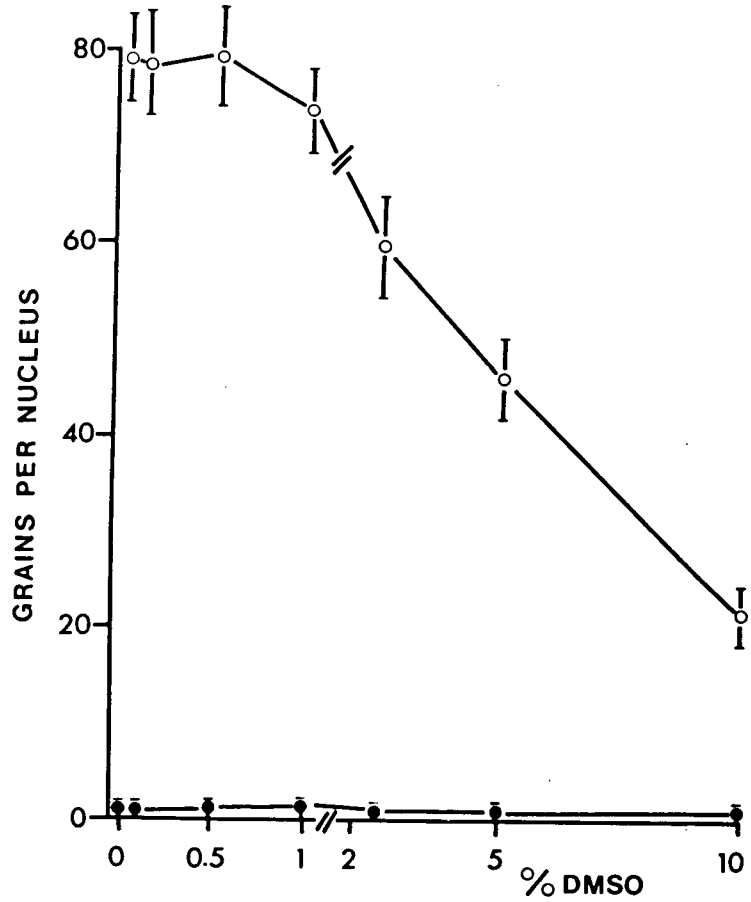

A
B
30
10
0
100
80
60
40
20
0
10^{-7}
10^{-5}
10^{-3}
CONCENTRATION (M)
GRAINS PER NUCLEUS

FIGURE 15

Effect of increased dimethylsulfoxide concentration on DNA repair synthesis in cultured RTG cells (○, experimental; ●, control) following ultraviolet light exposure (100 ergs/mm^2). Assay conditions: irradiated cells incubated with ^{3}HTdR for 6h at 25 C, emulsion-coated slides kept in light-tight boxes for 30 days. One standard deviation plotted on the mean grain counts.


GRAINS PER NUCLEUS
80
60
40
20
0
0
0.5
1
2
5
10
% DMSO

repair decrease may be the result of toxicity. The final DMSO concentration in the culture medium for experiments therefore did not exceed 1%.

e) Use of rainbow trout S9 in the repair assay

Because the metabolic specificity of fish S9 is unknown, two types of rainbow trout hepatic S9 were used to activate AFB_1 and establish a dose-response curve (Figure 16). Over the AFB_1 concentration range tested, both S9 types (ie. from Aroclor 1254 or crude oil extract injected fish) produced approximately the same level of DNA repair. The quantity of fish S9 required to activate AFB_1 and generate DNA repair was then optimized for the RTG cells (Figure 17). As the amount of repair synthesis was increased very little beyond 200 ul S9/ml of reaction mixture this S9 quantity was then routinely used. Rat S9 is also routinely used at this concentration in our laboratory for the DNA repair assay. Rat and fish S9 were compared for their ability to activate AFB_1 (at 25 C) and cause DNA repair in RTG and HF cells (Figure 18, 19). The two fish S9s were found to perform slightly better than the rat S9 with both cell lines, while the amount of repair with all S9s was greater in the HF cells.

f) DNA repair synthesis following treatment with PAHs

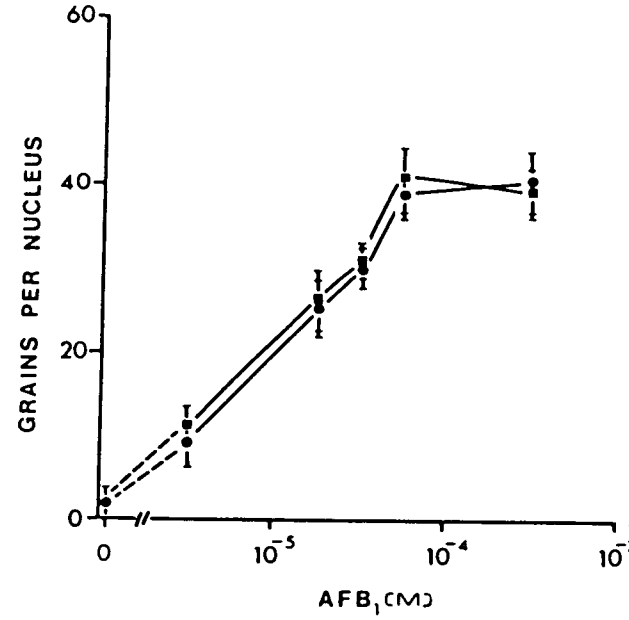
Polycyclic aromatic hydrocarbons are an important class of aquatic contaminants of which some, such as BP, DBA, and BA, are known carcinogens (National Academy of Sciences, 1972; IARC Monograph, 1973). DNA repair following treatment with PAHs was studied using S9 from rainbow trout to activate the test chemicals.

FIGURE 16

DNA repair synthesis in cultured RTG cells exposed to AFB_1 activated using rainbow trout S9 (■ , S9 from oil extract-injected fish; ● , S9 from Aroclor 1254-injected fish). Assay conditions: 25 C, emulsion-coated slides kept in light-tight boxes for 30 days. Mean grain counts plotted with a standard deviation on the mean.

FIGURE 17

DNA repair response in cultured RTG cells exposed to $1x10^{-4}M$ AFB_1 activated with increasing amounts of rainbow trout S9 (■ , S9 from oil extract-injected fish; ● , S9 from Aroclor 1254-injected fish). Assay conditions: 25 C, emulsion-coated slides kept in light-tight boxes for 30 days. Mean grain counts plotted with a standard deviation on the mean.


GRAINS PER NUCLEUS
60
40
20
0
0
10^{-5}
10^{-4}
10^{-3}
AFB_1 (M)

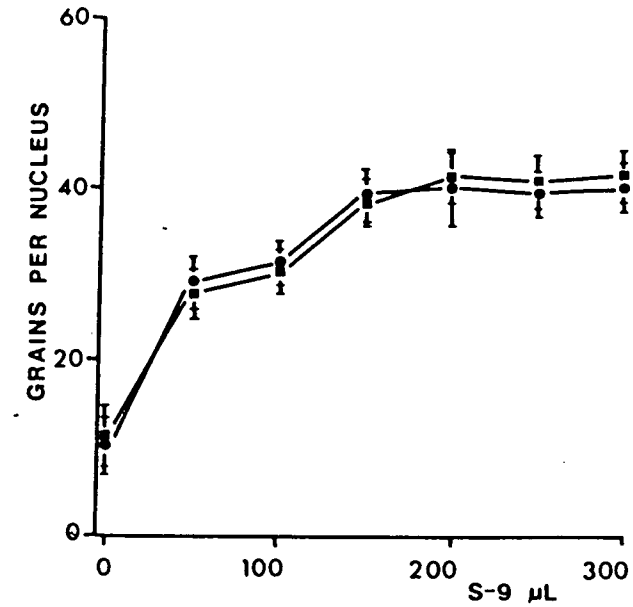
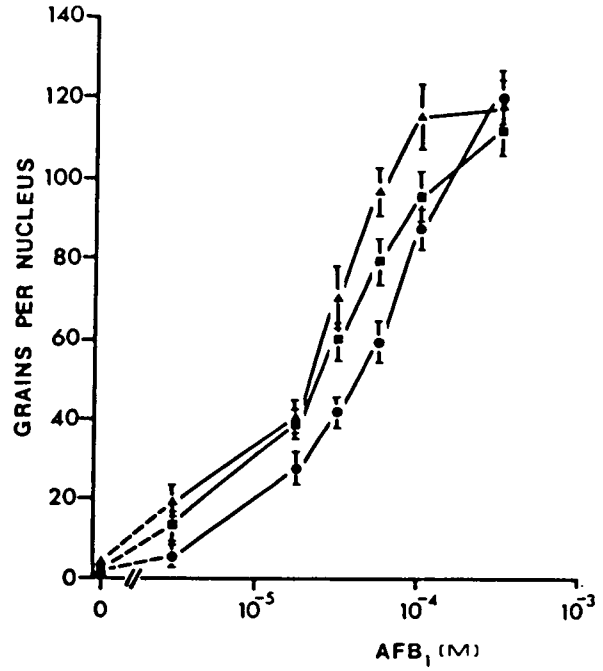

GRAINS PER NUCLEUS
60
40
20
0
0
100
200
300
S-9 µL

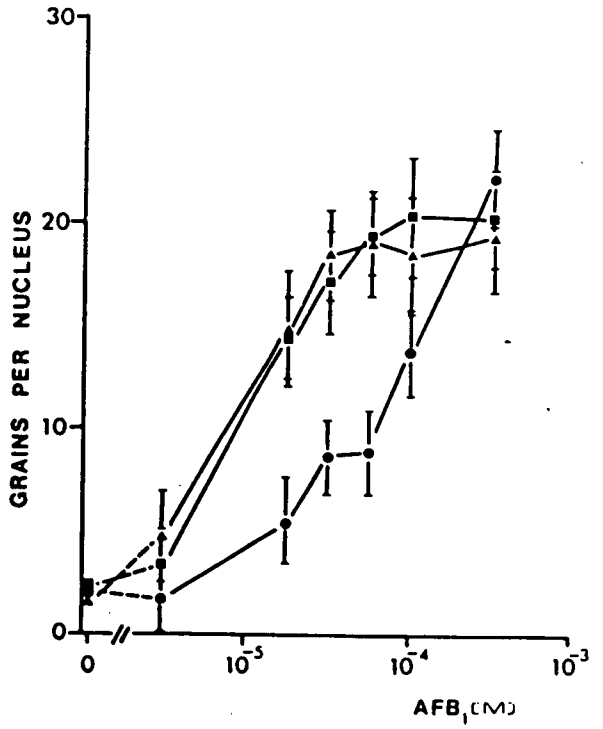
FIGURE 18

DNA repair response measured in cultured HF cells following exposure to AFB_1 activated with S9 from oil extract-injected rainbow trout (▲), Aroclor 1254-injected rainbow trout (■), or Aroclor 1254-injected rats (●). Assay conditions: 30 min test chemical treatment at 25 C was followed by a 3h incubation with ^{3}HTdR at 37 C, emulsion-coated slides kept in light-tight boxes for 18 days. Mean grain counts plotted with a standard deviation on the mean.

FIGURE 19

DNA repair in cultured RTG cells treated with AFB_1 activated with S9 from oil extract-injected rainbow trout (▲), Aroclor 1254-injected rainbow trout (■), or Aroclor 1254-injected rats (●). Assay conditions: 30 min test chemical treatment, followed by 3h incubation with ^{3}HTdR at 25 C. Emulsion-coated slides kept in light-tight boxes for 18 days. Mean grain counts plotted with a standard deviation on the mean.


GRAINS PER NUCLEUS
140
120
100
80
60
40
20
0
0
10^{-5}
10^{-4}
10^{-3}
AFB_1 (M)


GRAINS PER NUCLEUS
30
20
10
0
0
10^{-5}
10^{-4}
10^{-3}
AFB_1 (M)

The repair assay was first conducted with both HF and RTG cells using the original assay conditions in order to check the activating ability of trout S9 produced from fish injected with Aroclor 1254. HF cells exhibited the greatest levels of repair following treatment with BP, only a slight or no response after DBA or BA treatment, and no response following PY treatment (Figure 20). Only BP generated a small amount of repair in the RTG cells.

As only a very limited repair response was observed in the RTG cells, a second assay was conducted using the assay conditions found to enhance the repair response and both trout S9s, thus re-assessing the repair response and testing the influence of the different S9s (Figure 21). The greatest level of RTG cell repair was observed following treatment with BP activated with S9 from rainbow trout injected with Aroclor 1254. A lower level of repair was noted in RTG cells treated with BP activated with S9 from the rainbow trout injected with the oil extract. Both types of rainbow trout S9 elicited negligible amounts of repair in the RTG cells with DBA and BA treatments. As with the HF cells, no repair was observed after PY treatment.

g) Repair inhibition assay

Although DNA repair has been reported in mammalian cells following treatment with a variety of nitrosamines, mycotoxins, pesticides, and polycyclic aromatic hydrocarbons (Mitchell et al., 1983), other chemicals inhibit repair (Painter, 1981). When analyzing complex mixtures both DNA repair and inhibition assays can be conducted to achieve a

FIGURE 20

DNA repair synthesis in cultured RTG (● , PAH+S9; O , PAH only) and HF (■ , PAH+S9; □ , PAH only) cells exposed to (A) BP, (B) DBA, (C) BA, and (D) PY activated with S9 from Aroclor 1254-injected rainbow trout. Assay conditions: 30 min activated PAH treatment at 25 C followed with 3h ^{3}HTdR incubation at 25 C (RTG) or 37 C (HF), emulsion-coated slides kept in light-tight boxes for 18 days prior to developing. Mean grain counts plotted with a standard deviation on the mean.

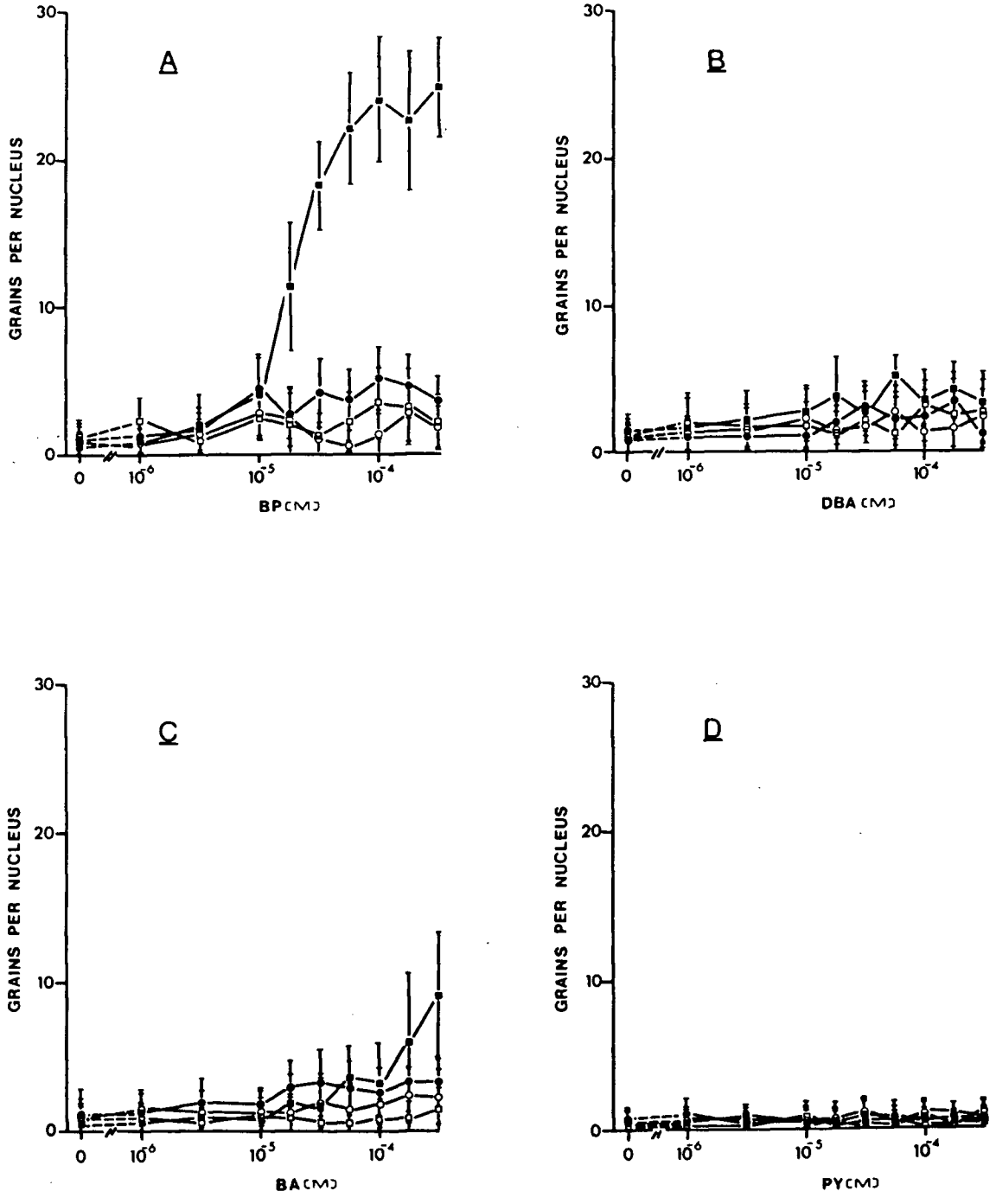
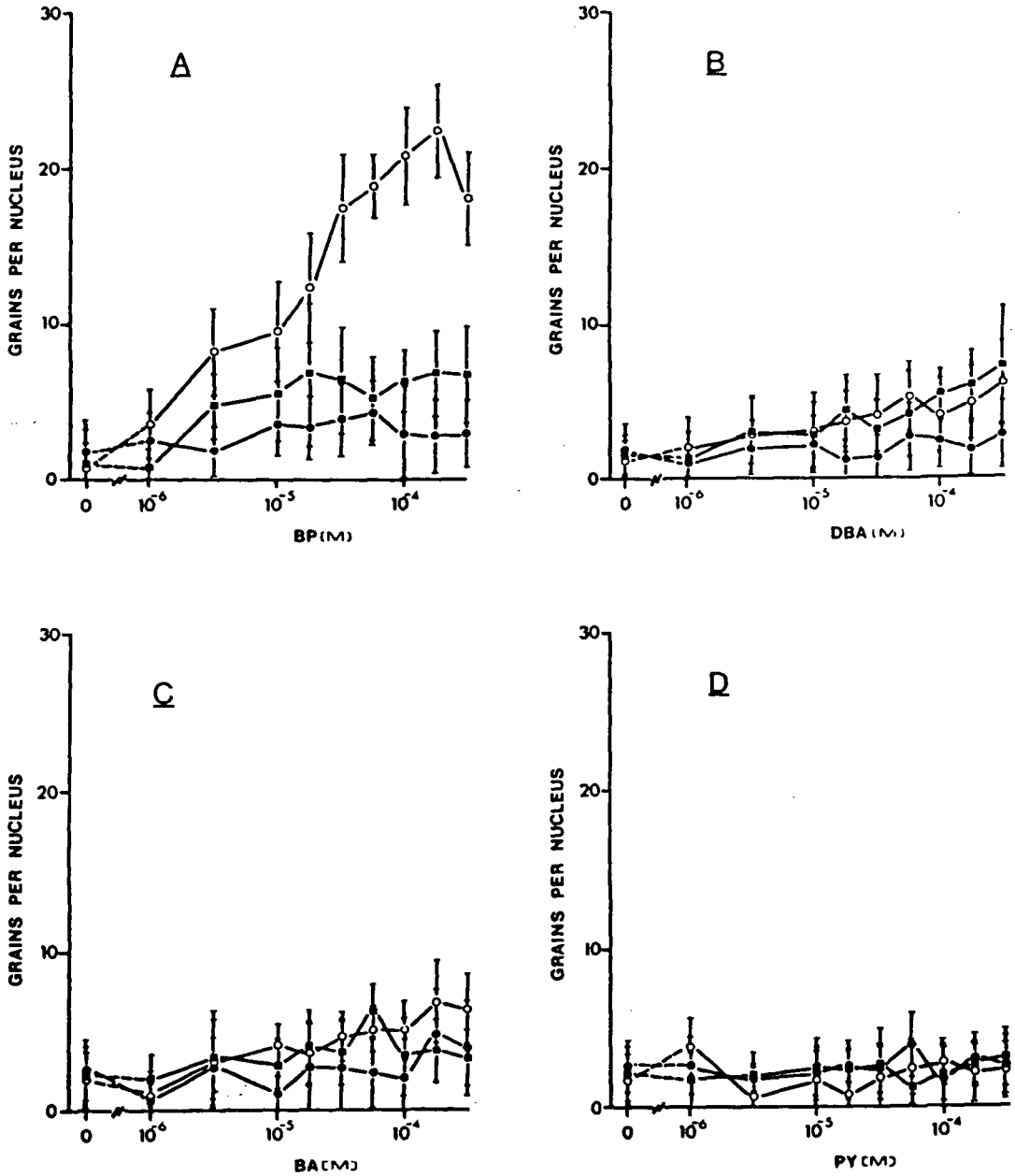

A
B
C
D
GRAINS PER NUCLEUS
30
20
10
0
0
10^{-6}
10^{-5}
10^{-4}
BP (M)
DBA (M)
BA (M)
PY (M)

FIGURE 21

DNA repair synthesis in cultured RTG cells following treatment with (A) BP, (B) DBA, (C) BA, and (D) PY activated with fish S9 (● , PAH only; O , PAH+S9 from Aroclor 1254-injected rainbow trout; ■ , PAH+S9 from oil extract-injected rainbow trout). Assay conditions: 30 min activated PAH treatment and 3h ^{3}HTdR incubation at 25 C, emulsion-coated slides kept in light-tight boxes for 30 days before developing. Mean grain counts plotted with a standard deviation on the mean.


A
B
C
D
GRAINS PER NUCLEUS
30
20
10
0
0
10^{-6}
10^{-5}
10^{-4}
BP(M)
DBA(M)
BA(M)
PY(M)

fuller understanding of the mixtures' effects. To avoid possible chemical interactions, the initial DNA damage is usually inflicted by ultraviolet light irradiation. This is possible with HF cells, since they do not repair the thymidine dimers by photoreactivation. But in fish cells this DNA damage is repaired by photoreactivation (Table 2) which limits this assay's usefulness.

2. DNA Repair Synthesis in Primary Cells Isolated From Rainbow Trout

The incorporation of S9 into the *in vitro* DNA repair and other genotoxicity assays is necessary as these cellular test systems usually have a reduced capability to metabolize test chemicals. How representative the *in vitro* results are of *in vivo* experiments has been questioned due to the possibility of artefacts being introduced by the S9 activation. As freshly isolated primary cells retain their ability to metabolize the test chemicals, no S9 is required, and no assay artefacts caused by the S9 are possible. It was also of interest to compare the amount of primary cell and *in vitro* RTG cell repair to see if the ability to repair DNA damage has been lost with longterm culturing of the RTG cells. Lastly an ultimate goal was to directly assess an aquatic environment's genotoxic burden. One means of doing this would be to collect fish from a suspect area, remove tissues of interest, isolate primary cells from them, and then determine the on-going amount of DNA repair.

TABLE 2

DNA repair in RTG cells irradiated with ultraviolet light, then incubated with ^{3}HTdR in light (under laboratory fluorescent and incandescent lights) or dark (no lights) conditions for 3 h at 18 C. And 18-day emulsion exposure period was used.

UV Dose ($ergs/mm^2$)	Grains per Nucleus ± Standard Deviation	
	Light Conditions	Dark Conditions
0	2 ± 1.6	1 ± 0.9
30	4 ± 2.3	10 ± 2.8
60	17 ± 3.6	25 ± 2.1
90	24 ± 3.7	31 ± 4.1

The experiments conducted here measured DNA repair in primary cells from rainbow trout liver, stomach, and intestine. Cell viability was estimated at 2 and 6h using the trypan blue exclusion technique. Observed mortality of the primary cells was generally less than 5 to 10% 6h after isolation. The level of DNA repair was greatest in primary liver cells for all 3 of the test chemicals used (Figure 22). The magnitude of their response was greatest with 4NQO and least with AFB_1. The stomach cells showed small amounts of repair in response to MNNG and 4NQO but failed to respond to AFB_1. Intestinal cells exhibited low levels of repair following 4NQO treatment only.

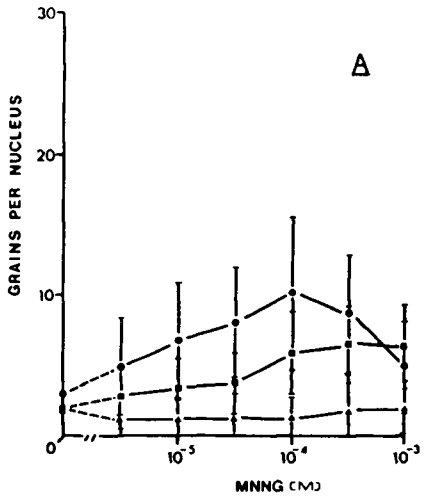
Preliminary primary cell DNA repair experiments failed to detect any repair in rainbow trout red blood cells following MNNG or 4NQO treatments.

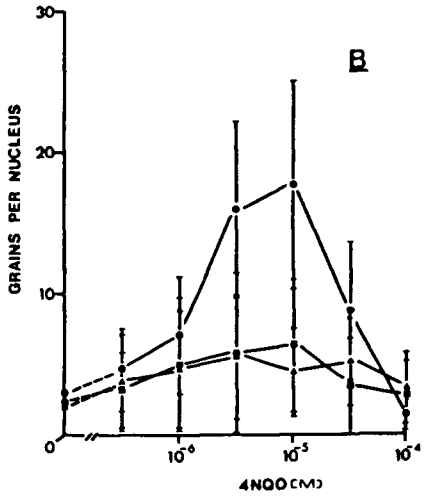
3. DNA Repair in Tissue Slices

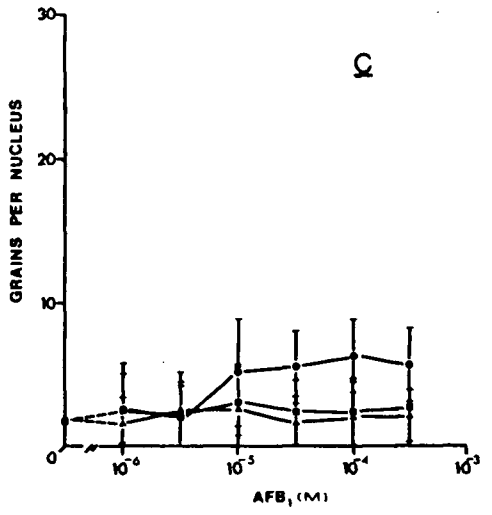
Another technique used which approximates <u>in vivo</u> treatments is to remove fish tissues, treat slices of them with ^{3}HTdR and test chemicals, and then to examine cells in tissue sections for evidence of DNA repair. As the initial DNA repair experiments indicated that different classes of organisms may possess different levels of DNA repair it was anticipated that the X-cells, if parasites, and epithelial cells in the flatfish epithelial papillomas could be distinquished (assuming the epithelial cells demonstrated considerable DNA repair and the X-cells little or none). The observations made indicated no increase in grain counts above

FIGURE 22

DNA repair synthesis in primary rainbow trout liver (●), stomach (■), and intestine (▲) cells exposed to (A) MNNG, (B) 4NQO, and (C) AFB_1. Assay conditions: 18 C, simultaneous treatment with the test chemical and ^{3}HTdR for 6h, emulsion-coated slides kept in light-tight boxes for 30 days. A standard deviation is plotted on the mean grain counts.


A
GRAINS PER NUCLEUS
30
20
10
0
10^{-5}
10^{-4}
10^{-3}
MNNG (M)


B
GRAINS PER NUCLEUS
30
20
10
0
10^{-6}
10^{-5}
10^{-4}
4NQO (M)


C
GRAINS PER NUCLEUS
30
20
10
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
AFB_1 (M)

baseline control levels following MNNG or 4NQO treatments. The X-cells within starry flounder epidermal papillomas had few grains while some of the surrounding epithelial cells were occasionally heavily grained (ie. in S-phase).

B. DNA Breakage Assays

1. DNA Breakage Assays with Cultured Cells

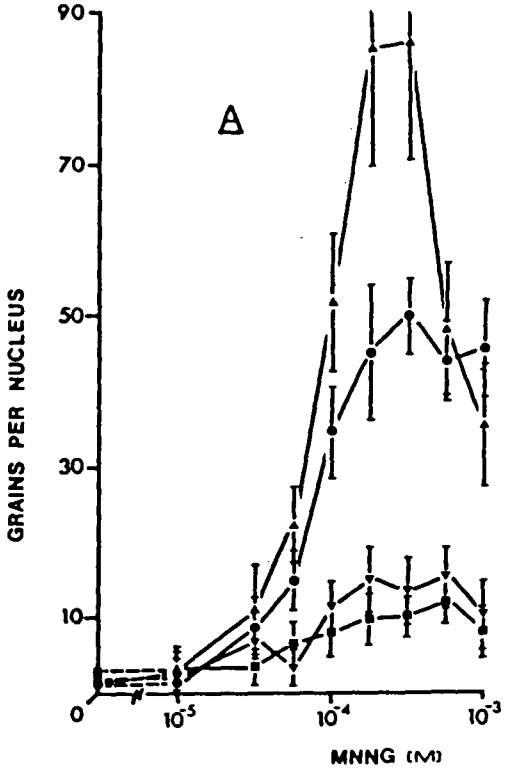
a) Cell line comparisons

A DNA repair response similar to the earlier cell line comparisons (Figure 2) was found with the Ul-F and Ul-H cells (Figure 23). Significant levels of repair were noted for the cell lines following treatment with both 4NQO and MNNG. As previously found, dose-dependent responses peaked at near the same concentration and the repair magnitude varied both with the cell line (HF > CHO > Ul-H = Ul-F) and the test chemical used (4NQO > MNNG).

Examination of preparations for chromosome aberrations is complicated by a reduced number of metaphase plates due to a delay in mitotic activity following both manipulation of the cells and, to an even greater extent, chemical treatment. The ^{3}HTdR labelling technique was used with dividing cultures to determine the duration of the mitotic delay after 4NQO treatment (Table 3). All of the cell lines demonstrated a reduced division rate in control preparations following the introduction of just fresh culture medium into the culture dish. The post-treatment metaphase frequency was dependent on the cell line and the chemical concentration since higher levels were more toxic. The largest number of metaphases were

FIGURE 23

DNA repair in U1-F (■) and U1-H (▼), CHO (●), and HF (▲) cells exposed to (A) MNNG and (B) 4NQO. Assay conditions: test chemical and ^{3}HTdR applied simultaneously for a 3h treatment period at 18 C (fish cells) or 37 C (mammalian cells), emulsion-coated slides kept in light-tight boxes for 18 days. Mean grain counts plotted with a standard deviation on the mean.


A
GRAINS PER NUCLEUS
90
70
50
30
10
0
10^{-5}
10^{-4}
10^{-3}
MNNG (M)

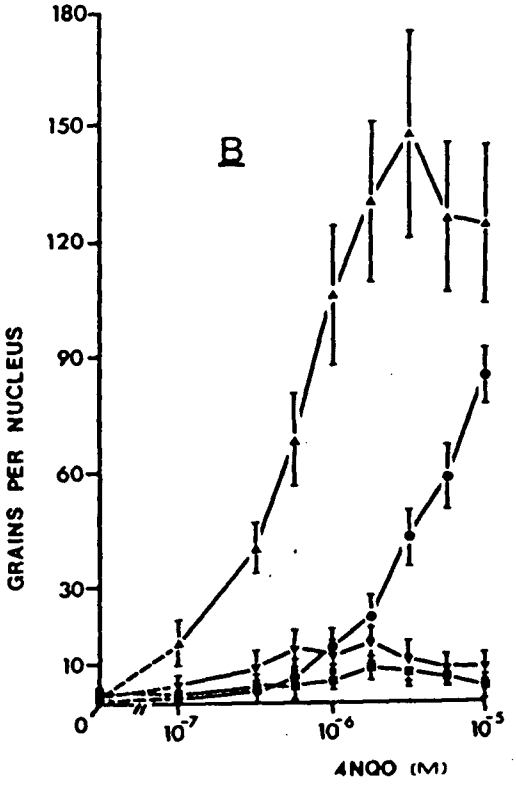

B
GRAINS PER NUCLEUS
180
150
120
90
60
30
10
0
10^{-7}
10^{-6}
10^{-5}
4NQO (M)

TABLE 3

Post-4NQO exposure ^{3}HTdR labelling of dividing Umbra limi fin and heart cells, Chinese hamster ovary cells, and human fibroblast cells. Assay conducted at 18 C (fish cells) or 37 C (mammalian cells) with an 18-day emulsion exposure period.

a) *Umbra limi* fin cells: number of heavily labelled nuclei per 1000 cells

	Post-Exposure Time Period (Hours)							
4NQO (M)	32-37	40-45	48-53	56-61	64-69	72-77	80-85	88-93
2.5×10^{-6}	3	2	14	18	24	-	15	5
1×10^{-6}	2	7	12	17	25	8	12	11
Control	2	8	18	23	8	9	9	8

b) *Umbra limi* heart cells: number of heavily labelled nuclei per 1000 cells

	Post-Exposure Time Period (Hours)						
4NQO (M)	4-8	8-12	12-16	16-20	20-24	24-28	28-32
1×10^{-6}	9	15	28	19	27	28	33
1×10^{-7}	9	22	74	75	44	22	25
Control	59	46	83	70	64	28	21

c) Chinese hamster ovary cells: number of heavily labelled nuclei per 1000 cells

	Post-Exposure Time Period (Hours)						
4NQO (M)	4-8	8-12	12-16	16-20	20-24	24-28	28-32
5×10^{-6}	7	7	17	36	34	64	37
1×10^{-6}	93	143	120	98	196	116	138
Control	113	121	118	167	201	85	145

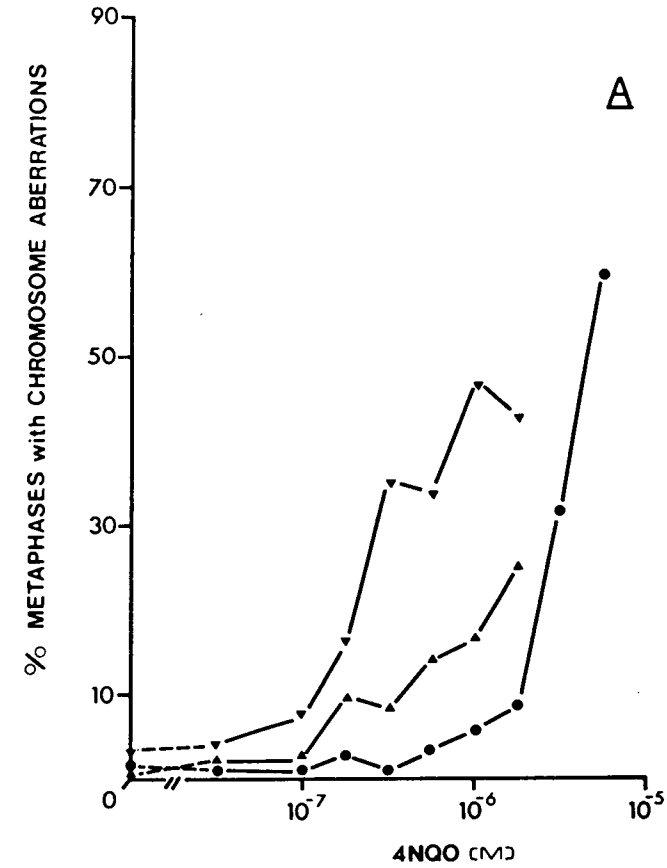
d) Human fibroblast cells: number of heavily labelled nuclei per 1000 cells

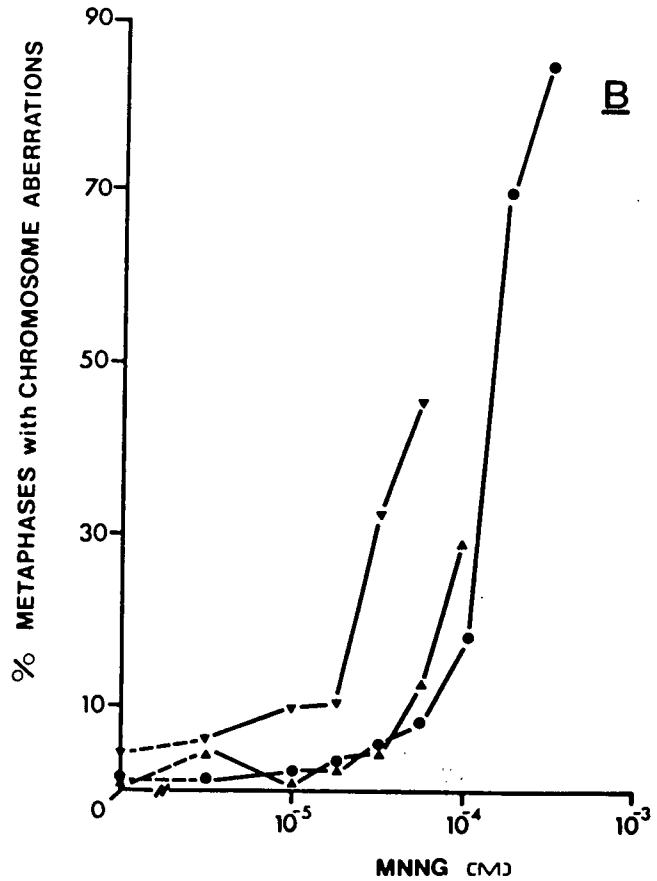
	Post-Exposure Time Period (Hours)						
4NQO (M)	16-20	20-24	24-28	30-34	34-38	38-42	42-46
1×10^{-6}	2	2	5	0	7	7	15
5×10^{-7}	2	6	3	13	4	15	28
Control	1	3	9	6	9	9	16

observed with the CHO cells. Waves of replicating CHO cells appeared by 12h with 10^{-6} M 4NQO, but sampling at 20 or 24h provided more metaphases over a broader concentration range. Similarly, with U1-H cells, by sampling between 16 to 24h after 4NQO treatment the mitotic delay effect could be reduced. The U1-F cells' mitotic delay was extremely long and only by sampling between 50 to 70h could a reasonable number of metaphases be expected. Experience with U1-F cells in culture indicated that their mitotic rate was very low. Previously HF cells were used in our laboratory for studying chromosome aberrations. Despite the low mitotic rate noted here and the 40 to 45h delay before sampling, earlier experiments with HF cells had successfully used a post-treatment sampling time of 30h. Having roughly established sampling times, initial experiments were designed to compare the frequency of chromosome aberrations in CHO, U1-H, and, despite their apparent low mitotic rate, HF cells. Significant dose-dependent frequencies of chromosome aberrations were observed in all 3 cell lines following treatment with MNNG or 4NQO (Figure 24). Mitotic inhibition at higher chemical concentrations varied according to the test chemical and the cell line. Mitotic inhibition/toxicity (ie. cell loss from coverslips) was noted above 7.5×10^{-5} M MNNG for U1-H, above 1×10^{-4} M MNNG for HF, and for both of these cell lines above 2.5×10^{-6} M 4NQO. The peak aberration frequency was dependent on the cell line (CHO > U1-H > HF), but the U1-H cells responded at slightly lower concentrations of both of the test chemicals.

FIGURE 24

Frequency of chromosome aberrations in U1-H (▼), CHO (●), and HF (▲) cells treated for 3h with (A) 4NQO and (B) MNNG.


A
% METAPHASES with CHROMOSOME ABERRATIONS
90
70
50
30
10
0
10^{-7}
10^{-6}
10^{-5}
4NQO (M)


B
% METAPHASES with CHROMOSOME ABERRATIONS
90
70
50
30
10
0
10^{-5}
10^{-4}
10^{-3}
MNNG (M)

In contrast to the chromosome aberration test where cells with a small number of large chromosomes are preferable, the micronucleus test can be applied to most dividing cell populations. In vitro comparisons were made with HF, CHO, U1-H, and U1-F cells following treatment with 4NQO or MNNG (Figures 25, 26). Since the appearance and frequency of micronuclei is dependent, in part, on the rate of cell division, which varies according to the particular cell line and may be delayed by mutagen exposure, the cells were sampled at 32h intervals up to 144h.

A significant increase in micronuclei frequency was not noted in any of the cell lines by 16h after 4NQO treatment (Figure 26). Overall, the frequency increased only marginally (2 to 3-fold) in U1-F and HF cells, but a significant number of micronuclei were found with both CHO and U1-H cells, with the CHO cells peaking at a slightly higher frequency. Both CHO and U1-H cells peak at approximately the same 4NQO concentration, but the maximum number of micronuclei occurred at 48h for CHO and 144h for U1-H cells. Since all the cell lines were exposed to all of the concentrations (5×10^{-8} to 5×10^{-6} M), it was noted that a mitotic inhibition/toxic response often occurred above $1 - 2.5 \times 10^{-6}$ M 4NQO, and that this effect became more apparent at the later sampling times.

As with 4NQO, the increase in the frequency of micronuclei following exposure of U1-F and HF cells to MNNG was marginal (Figure 25), showing only a 2 to 3-fold increase over controls at the sampling times. U1-H and CHO cells again peak at approximately the same concentration (5×10^{-4} M

FIGURE 25

Frequency of micronuclei in (A) U1-F, (B) U1-H, (C) CHO, and (D) HF cells measured at 16h (●), 48h (■), 80h (▲), 112h (▼), and 144h (○) following 3h treatment with MNNG.

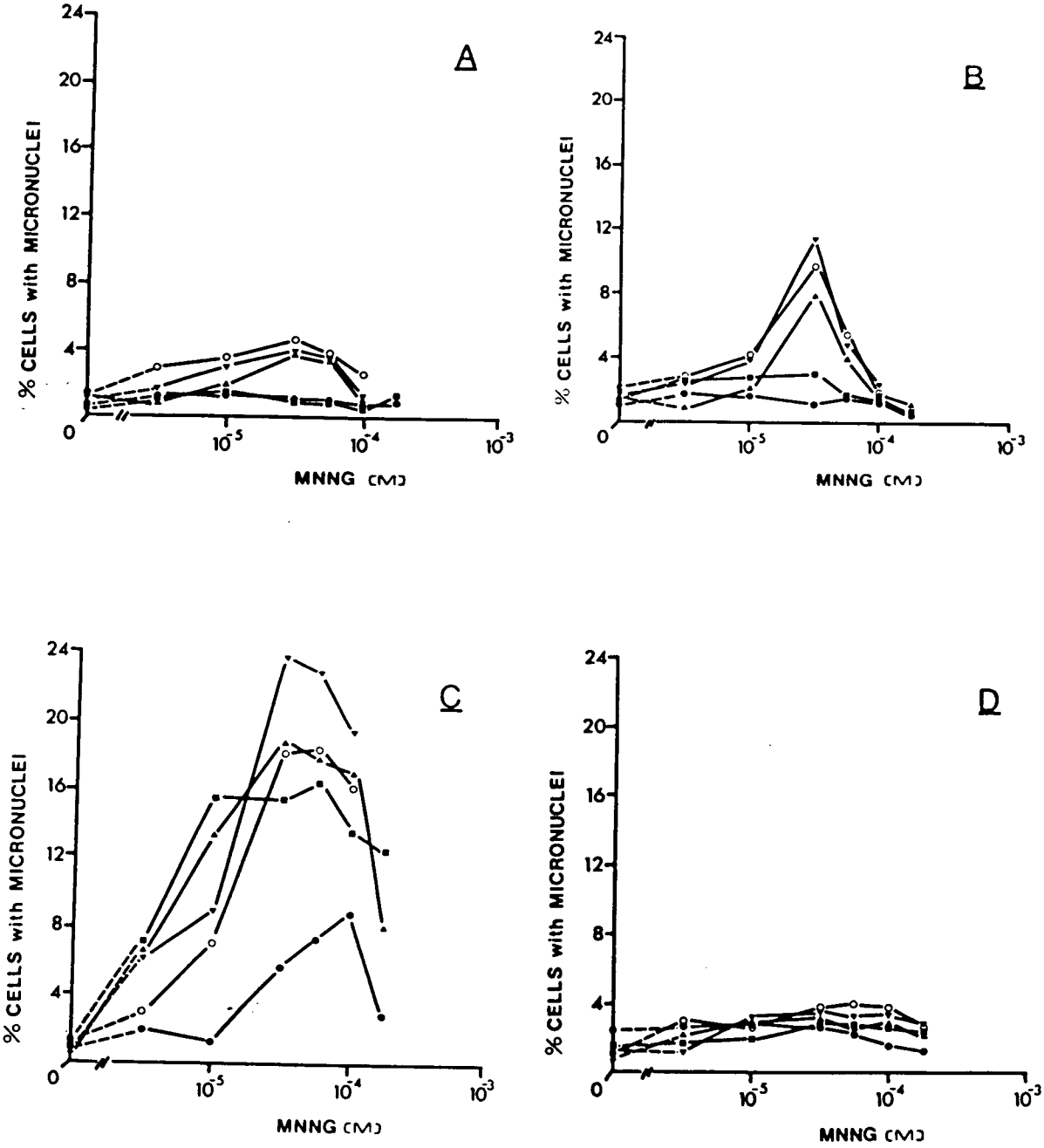
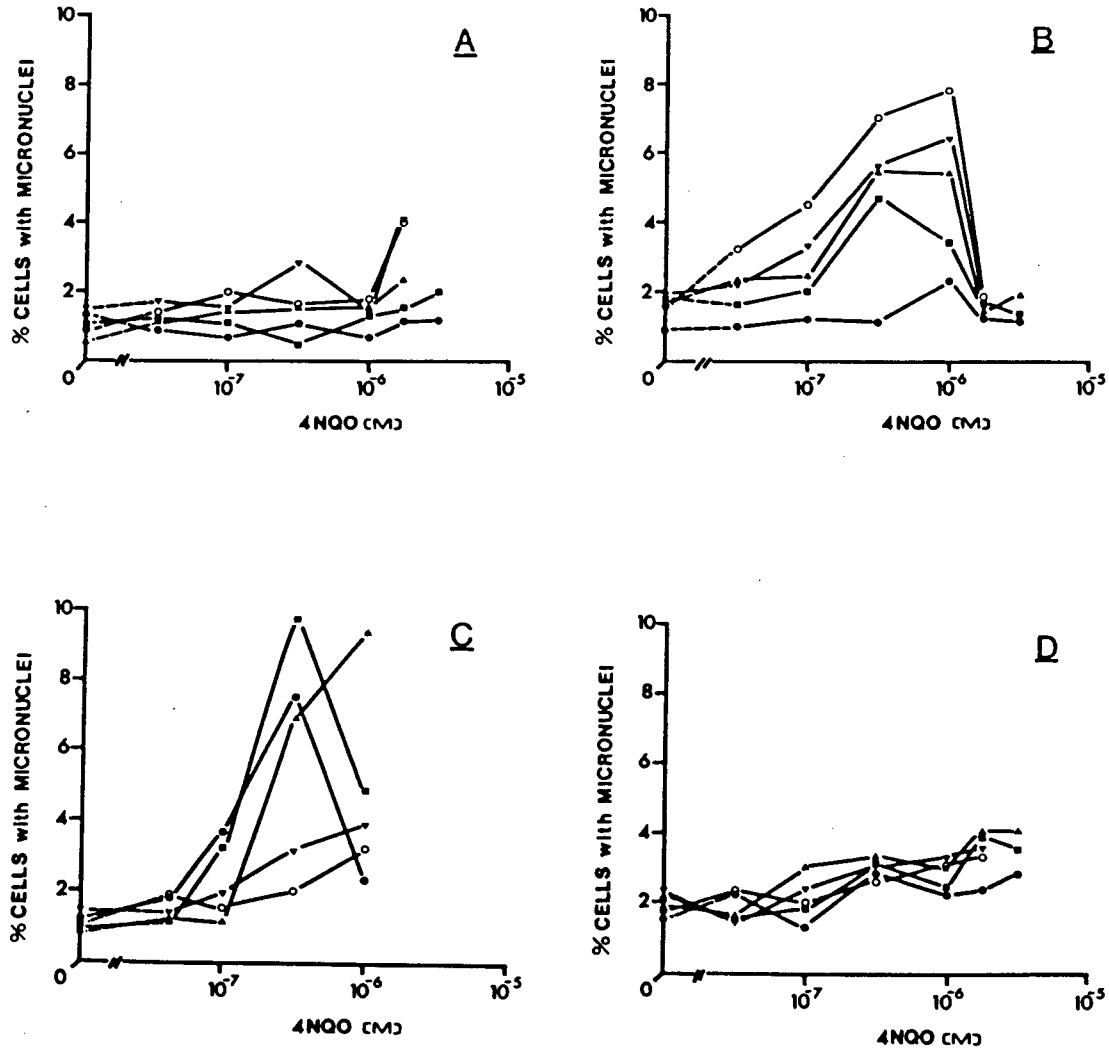

A
B
C
D
% CELLS with MICRONUCLEI
MNNG (M)
0
4
8
12
16
20
24
10^{-5}
10^{-4}
10^{-3}

FIGURE 26

Frequency of micronuclei in (A) U1-F, (B) U1-H, (C) CHO, and (D) HF cells measured at 16h (●), 48h (■), 80h (▲), 112h (▼), and 144h (O) following 3h treatment with 4NQO.


A
B
C
D
% CELLS with MICRONUCLEI
4NQO (M)
0
2
4
6
8
10
10^{-7}
10^{-6}
10^{-5}

MNNG), the magnitude of response being greater in the CHO cells. The maximum micronuclei frequency following MNNG treatment occurred at 112h for both CHO and U1-H. Since all of the cell lines were exposed to the full MNNG concentration range (5×10^{-8} to 5×10^{-6} M), the mitotic inhibition/toxic response noted at the higher concentrations becomes more apparent at the later sampling times.

b) Examination of the effect of post-exposure duration and DMSO concentration on chromosome aberration frequency

Prior to further chromosome aberration experiments with CHO and U1-H cells two assay details were examined. The aberration frequency was examined at intervals following exposure to 4NQO (Table 4). As previously noted, the U1-H cells respond at a lower concentration, but the peak aberration frequency was lower than in CHO cells. Aberrations can be detected at several concentrations after 12h with variation in frequency at particular concentrations between 16 to 24h. Beyond 24 to 28h the aberration frequency at lower chemical concentrations decreases, perhaps as a result of continuing DNA repair. From the 4NQO model it follows that sampling would be best performed between 16 to 24h after chemical treatment.

As in the DNA repair experiments, DMSO was used to dissolve the test chemicals. The effect of DMSO on aberration frequency was examined in activated AFB_1 treated CHO and U1-H cells (Table 5). At concentrations up to 4% DMSO there was no effect on CHO chromosome aberrations. Mitotic inhibition/toxicity with U1-H cells was observed with 4% DMSO

TABLE 4

Frequency of chromosome aberrations in Umbra limi heart and Chinese hamster ovary cells exposed to 4NQO and examined at intervals thereafter (percent metaphase plates with chromosome aberrations).

	4NQO (M)	Hours Post-Exposure				
		12	16	20	24	28
Chinese hamster ovary cells	1×10^{-5}	MI/T*	MI/T	MI/T	MI/T	MI/T
	5×10^{-6}	MI/T	51	55	65	55
	2.5×10^{-6}	25	22	13	23	9
	1×10^{-6}	12	3	3	2	0
	1×10^{-7}	2	2	0	2	0
	Control	1	1	1	0	0
Umbra limi heart cells	5×10^{-6}	MI/T	MI/T	MI/T	MI/T	MI/T
	2.5×10^{-6}	MI/T	43	46	45	MI/T
	1×10^{-6}	8	35	44	48	44
	1×10^{-7}	5	14	9	13	9
	5×10^{-8}	3	5	8	4	4
	Control	0	1	4	2	3

*MI/T = mitotic inhibition/toxicity.

TABLE 5

Effect of DMSO on chromosome aberration frequency in Umbra limi heart and Chinese hamster ovary cells exposed to rainbow trout S9 activated aflatoxin B_1 (percent metaphase plates with chromosome aberrations).

	AFB_1 (M)	Percent DMSO			
		0.1	1	2	4
Umbra limi heart cells	5×10^{-8}	43	40	-	MI/T*
	1×10^{-8}	23	28	28	MI/T
	Control	0	3	0	MI/T
Chinese hamster ovary cells	1×10^{-5}	83	88	83	80
	5×10^{-6}	85	90	80	76
	Control	2	1	0	2

*MI/T = mitotic inhibition/toxicity.

only. Further experiments therefore used a final DMSO concentration of not greater than 1%.

c) Chromosome aberrations following PAH treatment

Chromosome aberration experiments parallel to the DNA repair assays with PAHs were conducted. Up to 40 and 50% chromosome aberrations were observed in Ul-H and CHO cells respectively, following treatment with BP activated by fish S9 (Figure 27). This positive response was detected at or just below the concentration found to result in DNA repair. Few chromosome aberrations were detected in either cell line following DBA, BA, or PY treatment.

C. Field Testing the DNA Repair and Breakage Assays

1. Sediment Extracts

a) DNA repair

Repair assays were conducted with RTG and HF cells using Aroclor 1254-injected rainbow trout S9 to metabolize the extracts (Table 6). RTG and HF grain counts for cultures exposed to extracts from the Spanish Bank background area were little different from the counts for cells treated with extracts from the contaminated Sturgeon Bank area. In these experiments no toxic effects were noted following treatment with the Spanish Bank sediment extracts. Toxicity was also not evident after treatment of the HF cells with the Sturgeon Bank sediment extracts. Loss of RTG cells from the coverslips, indicating toxicity, was observed with Sturgeon Bank sediment extract treatment. This toxic effect was

FIGURE 27

Frequency of chromosome aberrations in Ul-H (● , with Aroclor 1254-injected rainbow trout S9; O , no S9) and CHO (▲ , with Aroclor 1254-injected rainbow trout S9; △ ,no S9) cells treated with (A) BP, (B) DBA, (C) BA, and (D) PY. 20 C 3h chemical treatment followed by a 20h recovery period at 18 C (Ul-H) or 37 C (CHO) prior to harvesting.

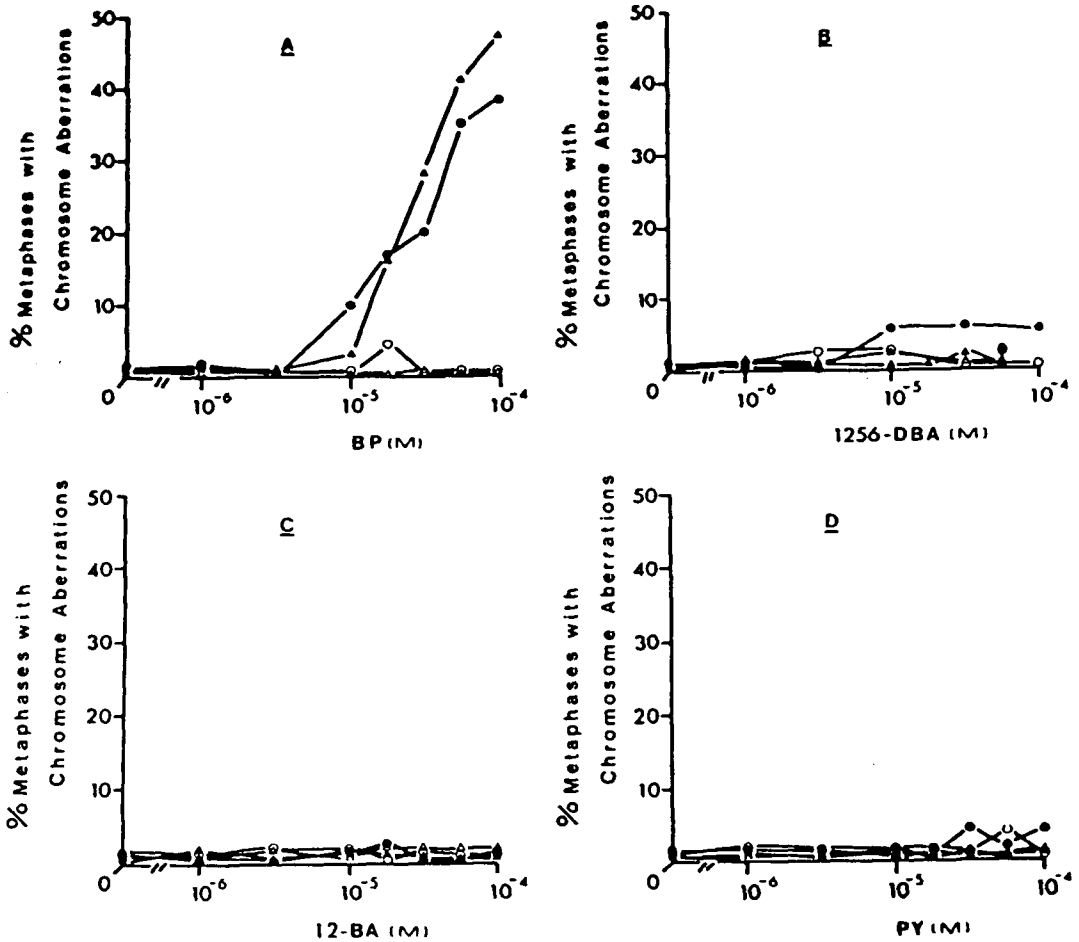

A
B
C
D
% Metaphases with Chromosome Aberrations
50
40
30
20
10
0
10^{-6}
10^{-5}
10^{-4}
BP (M)
1256-DBA (M)
12-BA (M)
PY (M)

TABLE 6

DNA repair synthesis in human fibroblast and rainbow trout gonad cells treated with sediment extracts from Spanish Bank and Sturgeon Bank (mean grain count ± standard deviation). Activation provided using Aroclor 1254-injected rainbow trout S9.

A. DNA repair in human fibroblast cells (assay temperature, 22°C; 3 hr extract: ^{3}HTdR treatment; 18-day emulsion exposure period)

1. Spanish Bank

Dilution	Spanish Bank #1		Spanish Bank #2		Spanish Bank #3	
	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9
1.0 : 10^2	1±1.1	1±1.0	1±1.4	1±1.0	1±1.5	1±1.1
7.5 : 10^3	1±1.2	1±0.8	1±1.6	1±1.5	1±1.4	1±1.2
5.0 : 10^3	1±0.9	1±0.8	1±1.4	1±1.0	1±1.1	1±1.1
2.5 : 10^3	1±1.3	1±0.6	1±1.1	1±2.2	1±1.2	1±0.8
1.0 : 10^3	1±1.4	1±1.9	2±2.4	1±1.1	1±1.5	1±1.4
7.5 : 10^4	1±1.4	1±0.9	1±1.3	1±1.1	1±1.0	1±0.8
5.0 : 10^4	1±1.2	1±1.2	1±1.3	1±1.3	1±1.5	1±1.2
2.5 : 10^4	1±1.1	1±1.4	1±1.0	1±1.3	1±1.0	1±1.1
1.0 : 10^4	1±1.2	1±1.0	1±1.3	1±1.3	1±1.2	1±1.6
5.0 : 10^5	1±0.8	1±1.2	1±1.8	1±1.1	1±1.2	1±1.1
1.0 : 10^5	1±1.1	1±1.0	1±1.5	1±1.1	2±2.4	1±1.4
Control	1±1.0	1±1.2	1±1.1	1±1.3	1±0.9	1±1.5

Table 6 (cont'd)

2. Sturgeon Bank

	#1		#2		#3		#4		#5	
Dilution	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9
1.0 : 10^2	1±1.8	1±1.3	2±1.9	1±1.3	1±1.6	1±1.2	2±1.8	1±1.2	2±1.6	1±1.2
7.5 : 10^3	1±1.3	1±1.2	2±1.8	1±1.8	1±1.3	1±1.2	2±2.4	1±1.6	2±2.0	1±1.0
5.0 : 10^3	1±1.2	1±0.9	1±1.5	1±1.6	1±1.2	1±1.2	2±1.7	1±1.5	2±1.7	1±1.0
2.5 : 10^3	2±1.7	1±1.3	1±1.2	1±1.3	2±1.7	1±1.3	2±1.7	1±2.0	2±1.7	1±1.2
1.0 : 10^3	1±0.9	1±1.5	1±1.5	1±1.0	2±1.6	2±1.6	2±1.6	1±1.7	2±1.8	1±1.3
7.5 : 10^4	1±1.3	1±1.1	1±1.0	1±1.5	2±1.7	1±1.3	1±1.3	1±1.5	1±1.4	1±0.8
5.0 : 10^4	1±1.6	1±1.4	1±1.7	1±0.8	2±1.4	1±1.3	1±1.4	1±1.2	2±1.5	1±1.0
2.5 : 10^4	1±1.4	1±0.9	1±1.1	1±1.0	1±1.3	1±1.3	2±1.6	1±1.0	1±1.5	1±1.3
1.0 : 10^4	1±1.2	1±1.1	1±1.8	1±1.9	1±1.6	2±1.7	2±1.3	1±1.6	1±1.6	1±1.4
5.0 : 10^5	1±1.4	1±0.8	1±0.8	1±1.0	1±1.5	1±1.3	2±1.3	1±1.3	1±2.0	1±1.4
1.0 : 10^5	1±1.6	1±1.1	1±1.3	1±0.9	1±1.4	1±1.6	1±1.2	1±1.1	1±1.4	1±1.1
Control	1±1.3	1±0.7	1±1.0	1±1.6	1±1.3	1±0.8	2±2.0	1±1.9	2±1.8	2±2.0

Table 6 (cont'd)

B. DNA repair in rainbow trout gonad cells (assay temperature, 22°C; 6 hr extract: ^{3}HTdR treatment; 30-day emulsion exposure period)

1. Spanish Bank

	Spanish Bank #1		Spanish Bank #2		Spanish Bank #3	
Dilution	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9
1.0 : 10^2	3±2.6	1±1.2	2±1.6	2±1.4	2±2.2	-
7.5 : 10^3	2±1.6	1±1.4	2±2.1	2±1.7	2±2.0	2±1.7
5.0 : 10^3	1±1.4	1±1.3	2±1.6	2±1.8	2±1.6	-
2.5 : 10^3	1±1.1	1±1.0	2±1.7	2±1.6	2±1.9	2±1.9
1.0 : 10^3	1±1.1	1±1.4	1±1.6	2±2.0	2±2.2	2±1.4
7.5 : 10^4	2±1.8	1±1.9	2±2.1	2±1.7	3±2.1	2±2.1
5.0 : 10^4	1±1.7	2±1.4	1±1.5	2±2.1	2±1.8	2±2.1
2.5 : 10^4	2±1.4	-	1±1.3	-	2±1.7	3±1.9
1.0 : 10^4	1±1.3	1±0.9	2±1.8	1±1.4	-	3±2.0
5.0 : 10^5	2±2.2	1±1.3	2±2.2	2±1.7	2±1.5	2±2.2
1.0 : 10^5	1±1.1	1±1.2	1±1.8	3±2.1	2±1.7	2±2.1
Control	2±1.8	1±1.4	2±2.3	-	3±2.3	-

Table 6 (cont'd)

2. Sturgeon Bank

	#1		#2		#3		#4		#5	
Dilution	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9
1.0 : 10^2	**	1±1.0*	1±1.5*	1±1.3	**	**	2±1.6*	1±1.0	3±2.1	1±0.9
7.5 : 10^3	**	1±1.0*	1±1.2*	1±1.0	**	1±1.4	3±1.9*	1±1.0	2±2.0	2±1.7
5.0 : 10^3	1±1.3*	1±1.0*	2±1.2*	1±0.8	2±1.4*	1±0.9	1±0.9*	1±1.6	2±1.9	1±1.1
2.5 : 10^3	2±1.5	1±1.4	1±1.2*	1±1.0	1±1.5	1±1.0	3±2.0	2±2.1	2±1.7	2±1.5
1.0 : 10^3	1±1.1	1±1.1	2±1.3*	1±1.1	1±1.6	1±1.2	2±2.4	1±1.5	2±2.0	1±1.6
7.5 : 10^4	2±1.7	1±1.1	1±1.3	1±0.9	1±1.6	1±1.7	3±1.9	2±1.4	2±2.0	1±1.7
5.0 : 10^4	2±1.4	1±0.8	1±1.2	1±0.9	1±1.2	1±0.7	1±1.3	2±1.6	2±1.7	2±1.4
2.5 : 10^4	1±1.2	1±1.1	1±1.1	1±1.0	1±1.3	1±1.5	2±2.3	2±1.6	2±1.9	1±1.5
1.0 : 10^4	1±0.7	1±1.1	1±1.5	1±1.0	2±1.9	1±1.6	2±1.7	2±1.9	2±2.0	1±4.0
5.0 : 10^5	1±1.3	1±1.1	1±0.9	1±1.1	2±1.5	1±1.4	2±2.0	2±2.4	2±2.4	2±1.5
1.0 : 10^5	1±0.7	1±1.2	1±1.0	1±1.8	1±1.1	2±1.3	2±1.5	2±1.8	2±1.9	1±1.0
Control	1±1.1	1±1.0	1±1.4	1±0.7	2±1.8	1±1.5	3±2.4	3±2.0	1±1.1	1±0.9

*Slightly toxic.
**Toxic.

generally greater with extracts from sediments collected from locations closer to the point of discharge, but the effect was lessened with the presence of S9.

b) Chromosome aberrations

CHO and Ul-H metaphases were examined for chromosome aberrations following exposure to the sediment extracts. For CHO cells (Table 7) control preparations had an aberration frequency of 0 to 2% while the background aberration frequency from the Spanish Bank extracts varied 0 to 4%. Treatment with extracts from the contaminated Sturgeon Bank sediments resulted in a 4-fold increase in aberration frequency in CHO cells. The chromosome aberration frequency in Ul-H control preparations was generally 0 to 2%. Spanish Bank extract treated cells exhibited chromosome aberration frequencies ranging from 0 to 10%, with higher levels observed from treatment with extract #3, that from the collection location closest to Sturgeon Bank. Treatment with Sturgeon Bank sediment extracts yielded a 7 to 20-fold increase in aberration frequency over controls, depending on the extract and concentration.

Overall, more frequent cytotoxic effects were encountered with the Ul-H than the CHO cells. For the Spanish Bank extracts some toxic effects were noted with the extract closest to Sturgeon Bank. Toxic effects associated with the Sturgeon Bank extracts generally increased toward the sewage treatment plant's point of discharge but the effect was lessened by the presence of S9 in the treatment. Lower Ul-H aberration frequencies with extracts of sediments collected

TABLE 7

Frequency of chromosome aberrations in Chinese hamster ovary and Umbra limi heart cells treated with sediment extracts from Spanish Bank and Sturgeon Bank (percent metaphase plates with chromosome aberrations). Activation provided using Aroclor 1254-injected rainbow trout S9.

A. Chromosome aberrations in Chinese hamster ovary cells (assay temperature, 22°C, 3 hr extract treatment; post-exposure period temperature, 37°C)

1. Spanish Bank

	Spanish Bank #1		Spanish Bank #2		Spanish Bank #3	
Dilution	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9
$1.0 : 10^2$	2	0	3	1	0*	1
$7.5 : 10^3$	2	1	1	0	3*	1
$5.0 : 10^3$	1	1	3	2	1*	2
$2.5 : 10^3$	0	0	1	1	1*	0
$1.0 : 10^3$	0	1	1	1	2	0
$7.5 : 10^4$	0	1	2	2	1	0
$5.0 : 10^4$	0	0	1	2	0	0
$2.5 : 10^4$	1	0	4	3	2	1
$1.0 : 10^4$	2	1	2	0	1	1
$5.0 : 10^5$	0	4	1	2	2	0
$1.0 : 10^5$	2	2	1	2	3	1
Control	2	1	1	1	2	0

*Slightly toxic; decreased metaphase frequency.

Table 7 (cont'd)

2. Sturgeon Bank

	#1		#2		#3		#4		#5	
Dilution	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9
$1.0 : 10^2$	1	6	8*	7	**	2	4	1	0	0
$7.5 : 10^3$	6*	16*	4*	0	4*	2	4	3	2*	1*
$5.0 : 10^3$	**	**	2	2	1*	0	2	1	1*	0*
$2.5 : 10^3$	2	0	2	1	0*	0	1	0	5	1
$1.0 : 10^3$	2	0	0	2	0*	2	2	1	3	0
$7.5 : 10^4$	2	0	1	1	2	0	0	0	0	1
$5.0 : 10^4$	1	0	0	1	1	1	0	1	1	1
$2.5 : 10^4$	0	0	0	0	0	1	0	0	0	1
$1.0 : 10^4$	1	1	0	1	1	0	0	0	0	0
$7.5 : 10^5$	-	-	-	-	-	-	-	-	-	-
$5.0 : 10^5$	0	1	0	2	0	1	1	2	0	2
$2.5 : 10^5$	-	-	-	-	-	-	-	-	-	-
$1.0 : 10^5$	0	0	2	0	2	0	0	0	0	1
$5.0 : 10^6$	-	-	-	-	-	-	-	-	-	-
Control	0	1	0	2	1	0	1	0	0	0

*Slightly toxic; decreased metaphase frequency.
**Toxic/mitotic inhibition.

Table 7 (cont'd)

B. Chromosome aberrations in Umbra limi heart cells (assay temperature, 18°C; 3 hr extract treatment; post-exposure period temperature, 18°C)

1. Spanish Bank

	Spanish Bank #1		Spanish Bank #2		Spanish Bank #3	
Dilution	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9
1.0 : 10^2	6	2	0	4	10*	9
7.5 : 10^3	0	4	2	2	6	2
5.0 : 10^3	4	0	0	6	10	0
2.5 : 10^3	4	0	0	2	2	6
1.0 : 10^3	6	0	4	2	0	2
7.5 : 10^4	4	4	0	0	0	4
5.0 : 10^4	2	2	2	4	2	6
2.5 : 10^4	2	0	2	0	4	4
1.0 : 10^4	2	4	0	0	0	6
5.0 : 10^5	0	2	0	4	0	2
1.0 : 10^5	2	0	0	0	4	2
Control	4	0	2	0	0	-

*Slightly toxic; decreased metaphase frequency.

Table 7 (cont'd)

2. Sturgeon Bank

	#1		#2		#3		#4		#5	
Dilution	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9	(-)S9	(+)S9
1.0 : 10^2	**	**	**	**	**	**	**	23	6	1
7.5 : 10^3	**	**	**	8	**	15*	**	8	**	2*
5.0 : 10^3	**	**	**	8	**	8*	**	6	**	7*
2.5 : 10^3	**	**	**	4	**	**	40	2	**	10*
1.0 : 10^3	**	**	**	3	**	**	28	4	20	1
7.5 : 10^4	**	10	14	0	**	1	11	5	18	2
5.0 : 10^4	**	4	15	2	3	4	6	3	5	0
2.5 : 10^4	**	4	**	0	3	2	5	0	0	2
1.0 : 10^4	**	4	8	2	2	2	0	3	4	0
7.5 : 10^5	15	4	2	2	2	0	-	-	-	-
5.0 : 10^5	10	4	2	1	0	0	4	2	0	2
2.5 : 10^5	5	0	4	2	2	2	-	-	-	-
1.0 : 10^5	0	0	2	0	0	0	2	2	2	0
5.0 : 10^6	2	0	0	2	-	-	-	-	-	-
Control	0	2	0	0	0	0	0	0	0	0

*Slightly toxic; decreased metaphase frequency.
**Toxic/mitotic inhibition.

from closer to the discharge point, in conjunction with the increase in toxicity, tend to indicate a masking of the DNA damaging event by the toxicity.

2. Micronuclei in Fish Cells *In Vivo*

Cells from the mouth, blood, and liver of starry flounder (*Platichthys stellatus*) collected from Spanish and Sturgeon Banks were examined for the presence of micronuclei. The Sturgeon Bank fish were collected from an area of demonstrated genotoxic activity while the Spanish Bank collection area was shown to have no activity (Table 7). Despite this difference in genotoxic activity the results (Table 8) do not show a marked difference in micronuclei frequency.

TABLE 8

Frequency of micronuclei in buccal, liver, and red blood cells from starry flounder (Platichthys stellatus) collected from Spanich Bank and Sturgeon Bank (mean number of micronuclei per 1000 cells ± standard deviation; number of fish examined).

	Buccal Cells	Blood Cells	Liver Cells
Spanish Bank :	4.5 ± 2.3; 22	2.9 ± 1.8; 22	5.7 ± 2.3; 17
Sturgeon Bank :	5.6 ± 2.2; 25	2.8 ± 1.7; 24	4.6 ± 2.5; 21

DISCUSSION

The prime objective of the research reported in this thesis was to evaluate the potential use of DNA repair and breakage assays for assessing genotoxicity in the aquatic environment. Previous research in our Cancer Research Laboratory had examined the repair of DNA and generation of chromosome aberrations in both HF and CHO cells. The HF cells are now preferred for DNA repair assays while tests for chromosome aberrations are usually performed with CHO cells due to easier manipulation and better sensitivity of these cell lines in the respective assays. In attempts to develop assays with more relevance to aquatic organisms and their environment, fish cells were used in the assays and the results compared to these existing model test systems for DNA repair and breakage.

1. Why Test for Aquatic Genotoxicity?

It has been claimed that 60 to 90% of all cancers in man are causally related to chemical carcinogen exposure (Boyland, 1969; Epstein, 1974; Heidelberger, 1975). Many of these chemicals, including pesticides, halogenated solvents, polycyclic aromatic hydrocarbons, and aromatic amines, used by industrialized societies become discharged into and accumulate in the aquatic environment (Kraybill, 1977; Moore et al., 1980; Nelson et al., 1980; Payne and Martins, 1980; Van Hoof and Verheyden, 1981; Alink, 1982; Sato et al., 1983). Genotoxic activity may also arise from natural sources such as

mycotoxins (Payne and Martins, 1980), fecal material (Stich et al., 1980), wood (Dunn and Hanham, 1983), and decomposing matter (Payne and Martins, 1980). Additional chemical complexity and genotoxicity results from chlorination and ozonation of drinking and wastewaters (Payne and Martins, 1980; Alink, 1982; Saxena and Schwartz, 1979; Dolara et al., 1981; Payne and Rahimtula, 1981). It is therefore not surprising to find elevated tumor frequencies in fish populations inhabiting polluted environments (Brown et al., 1973; Pierce et al., 1978; Smith et al., 1979; Black et al., 1980; Black et al., 1982; Malins et al., 1984). The hypothesis of a causal relationship is further supported by the observation of tumour formation in fish following in vivo exposures to chemical carcinogens (Meyers and Hendricks, 1982) and by studies demonstrating the ability of fish to metabolize carcinogens to metabolites which both bind to fish DNA and exhibit mutagenic activity (Stott and Sinnhuber, 1978; Varanasi and Gmur, 1980; Ahokas et al., 1979; Varanasi et al., 1981; Balk et al., 1982; Shelton et al., 1983). Of the genotoxic agents used in my own studies here, AFB_1, BP, and MNNG have been found to cause tumors in fish (Meyers and Hendricks, 1982). Ultraviolet light has also been found to cause pathological effects on fish in fish farms (Bullock, 1982) and ultraviolet light irradiated fish cells injected into the Amazon molly (Poecilia formosa) resulted in a high incidence of thyroid tumors (Rosen, 1980).

The development of rapid, inexpensive assays for genotoxic activity has permitted the screening of many complex

mixtures in addition to pure chemicals contributing to the genotoxicity. A tiered testing system has evolved whereby chemicals found to be positive in simpler bacterial or yeast assays are reassayed in more complex but meaningful tests with cultured cells (Bridges, 1974; Flamm, 1974; De La Iglesia et al., 1980). Although mammalian cells are most commonly used, there is increasing interest in incorporating fish cells and metabolizing enzymes from fish tissues into these assays. Here the aims are to discover whether the mammalian assay results are generally valid, to determine fish cell sensitivity to genotoxic agents, to provide an assay with increased relevance to fish and the aquatic ecosystem, and ultimately to develop a technique to directly assess the carcinogenic/genotoxic burden of a particular aquatic environment.

Several fish cell lines are available from the American Type Culture Collection (Rockville, Maryland) or through the originators of a particular line (Wolf and Mann, 1980). Some fish cell lines, particularly the RTG line, are used for assessing toxicity (Marion and Denizeau, 1983; Denizeau and Marion, 1984; Bols et al., 1985) in addition to their more traditional fish viral disease applications. Rainbow trout are the subjects of considerable research in addition to being a test fish for aquatic carcinogenesis (Sinnhuber et al., 1977; Hendricks et al., 1980). Therefore because of their ready commercial availability and the already existing data base it is advantageous to incorporate the RTG cell line into genotoxicity assays where possible and practical.

2. The DNA Repair Assay as a Test for Aquatic Genotoxicity

a) The DNA repair response in mammalian versus fish cells

In mammalian systems the DNA repair assay, which measures the extent of excision repair of damaged DNA, has been used *in vivo* and *in vitro* to test a diverse range of potential genotoxic agents including pesticides, heavy metals, and polycyclic aromatic hydrocarbons (Mitchell et al., 1983). This assay has both theoretical and practical significance because failure to effect repairs to damaged DNA, as in the human genetic disorder xeroderma pigmentosum, can lead to an elevated tumor frequency (Cleaver and Bootsma, 1975; Kraemer et al., 1984).

The comparative approach undertaken here indicated that at comparable concentrations of MNNG, 4NQO, NA2AAF, and AFB_1, fish cells exhibit markedly less autoradiographically measured DNA repair than human or rodent cells (Figure 2). Woodhead et al. (1980) also noted low amounts of *in vitro* fish cell excision repair in ultraviolet-irradiated rainbow trout gonad and Amazon molly cells. Regan et al. (1983) compared the DNA repair capacity of primary fish cells from *Tautoga onitis* and *Tautogolabrus adspersus*, with that of a cultured human fibroblast cell line and found at least 10-fold less repair in the fish cells following treatment with ultraviolet light, 4-nitroquinoline-1-oxide, ethyl methanesulfonate, methyl methanesulfonate, and N-acetoxy-acetylaminofluorene. However, these differences are misleading as the comparison made was between primary fish cells and an established human cell line.

The results here (Figures 2, 14, 22) indicate that the measured DNA repair capacity is much lower in primary than in cultured cells. Furthermore, the magnitude of the repair response in mammalian and fish cells varies with different genotoxic agents (Figure 2, Table 1), as has also been noted by Regan et al. (1983). However, the relatively minor differences in the amount of repair measured in the different fish cell lines (Figure 3) do not confirm the results of Regan et al. (1983) who observed a varying amount of repair between a number of test chemicals that had been used to treat primary cells from two fish species. But since Regan et al. (1983) report results for only a specific concentration, not a dose response, this finding may be an artefact.

b) Explanations for the low DNA repair response of fish cells

Due to uncertainty with the comparative DNA repair results, experiments were conducted to determine if the low fish cell response resulted from delayed DNA repair, differential absorption of test chemicals, or less DNA target per cell.

The time course of repair (Figure 5) in RTG, CHO, and HF cells following MNNG or 4NQO treatment is approximately the same despite the difference in repair magnitude. Most of the observed repair was complete within 20h and peaked within 6h. Similarly Stich and San (1970) with hamster cells and Warren and Stich (1975) with human fibroblasts observed the repair response to peak within a few hours following chemical treatment. The gradual return to baseline repair levels suggests a variation in the accessibility or ease of repair of

various DNA lesions as noted by Kantor and Setlow (1981).

The composition of mammalian and fish cell membranes may differ, leading to differential chemical absorption. This problem was examined by measuring DNA repair in RTG cells irradiated with ultraviolet light (Table 1), which is not dependent on cellular absorption. Results paralleling those of the chemical genotoxin treatments were found, with the DNA repair response varying HF > CHO > RTG.

Lehninger (1975), in summarizing DNA quantities per cell indicates 3-fold less DNA in fish than in mammalian cells, which could account for the low fish cell DNA repair response. However, review of more detailed compilations (Sober et al., 1968; Altman and Katz, 1976) suggests this is an oversimplification as some fish species, such as the rainbow trout, are listed as having DNA quantities per cell which approximate that of mammalian cells. The variation in measurements for the same species suggests that some of the differences may be due to the techniques used. DNA quantitative measurements made here showed approximately 10-15% less DNA per cell for the fish cells. These measurements were made using the same staining technique throughout and assaying all cell lines simultaneously. Sober et al. (1968) indicate also that the distribution of purines and pyrimidines is remarkably similar among most fish and mammalian species, so that grossly, mammalian and fish DNA's are quite similar. Thus the low fish cell repair response cannot be a consequence of a delay in the repair of DNA lesions, the lack of test chemical uptake, or greatly reduced DNA target.

Other factors may be important in interspecific comparisons of the levels of DNA repair. The assay measures excision repair which is an enzymatically mediated process. The quantitative activity of DNA polymerase, a key enzyme in the repair mechanism, has been measured in mitochondria and the activity was found to be greatest in human cells, less in rodent cells, and least in fish cells (Scovassi et al., 1979). Damage to DNA has also been found to be a nonrandom process, indicating a site-specificity of genotoxic agents (Meyne et al., 1979; Ornstein and Rein, 1979; Werner et al., 1981; Perin-Roussel, 1984). Possibly fish DNA simply has fewer sites for genotoxin attack. Regan et al. (1983) noted that in primary fish cells or cultured human cells treated with methyl methanesulfonate, N-acetoxy-acetylaminofluorene, or 4-nitroquinoline-1-oxide or irradiated with ultraviolet light that the repair patches were of approximately the same size but the number of repaired sites was usually greater in the mammalian cells. Preferential binding of genotoxic agents has been found in association with linker versus nucleosomal DNA (Jack and Brookes, 1982), satellite DNA versus the main DNA component (Melchior and Beland, 1984), and transcriptionally active versus inactive DNA areas (Yu, 1983). Site selectivity is also influenced by pH (Lyle et al., 1980; Chen, 1984). Further study will be required to determine the importance of these factors which may vary depending on the species of fish, or cell line, examined and the particular test chemical.

c) Enhancing the measured fish cell DNA repair response

When the unexpectedly low fish cell repair response first

became apparent in 1978, corroborative research (Woodhead et al., 1980; Regan et al., 1983) had not been published, so that several assay parameters were investigated to determine if the observed results were only a technique artefact. Another goal of this exercise was to determine ways of altering assay conditions to increase the measured fish cell repair response, thereby increasing the assay's usefulness as a technique to monitor the aquatic environment for genotoxicity.

Varying four assay parameters did result in increases in the measured DNA repair response. A longer ^{3}HTdR incubation time (Figure 6) allows more DNA to be repaired, hence more isotope is incorporated. As the greatest amount of repair occurs within a few hours of the test chemical treatment (Figure 5), incremental gains from increasing the treatment time decrease. Increasing the assay temperature to 25 C from 18 C (Figure 8) increases the repair response, probably through effects on the repair enzymes, rather than on membrane composition changes affecting permeability (Hazel and Prosser, 1974; De Torrengo and Brenner, 1976). 25 C is the upper tolerance limit for RTG cell growth (Plumb and Wolf, 1971). As some karyorrhexic nuclei were noted in RTG stock cultures maintained at 25 C for a prolonged period of time perhaps the DNA changes its conformation to a more unstable form or to one which is more open to genotoxic attack. Damage by 4NQO appears essentially to be immediately upon treatment, with little measurable gain beyond 30min (Figure 9). Lastly, a longer exposure period to the emulsion (Kodak NTB-3) permits

more of the incorporated ^{3}HTdR to degrade (Figure 7). As ^{3}HTdR has a half-life of 12.3 years (Rogers, 1979) the incremental response increases are roughly proportional.

Other assay parameters were tested, none of which enhanced the RTG repair response. Since the nutritional requirements of RTG cells are not clearly defined, the effects on DNA repair from holding the cells in arginine-deficient culture medium, which was also 7.5% lower in serum, were evaluated (Figure 10). No changes in the repair level were noted in cells held up to 7 days in ADM with 2.5% serum prior to treatment with MNNG. Warters et al. (1985) reported decreased DNA damage repair in cultured mammary carcinoma cells which were not "fed" (ie. culture medium was not changed prior to X-irradiation). The approach taken here (Figure 11) was to raise the nutrient level via the ADM serum concentration. No effect on the repair level was noted from varying the ADM serum concentration. As excision repair involves the incorporation of nucleotides from cellular pools it is critical to the assay to flood the pools with isotope. ^{3}HTdR is not limiting in RTG cell DNA repair, as increases in concentration did not increase repair grain counts (Figure 12). It is also unlikely that the pool ^{3}HTdR available becomes diluted as the ^{3}HTdR is added to excess and Das et al. (1983) found that expansion of Chinese hamster nucleotide pools required 2 to 4 hours after MNNG treatment. That there is adequate ^{3}HTdR penetration into the RTG cells is also supported by the existence of a few heavily-grained nuclei (ie. in S-phase) in control preparations. Several researchers

have examined variations in DNA repair capability with cell aging. Hart and Setlow (1976) with human cells and Ben-Ishai and Peleg (1974) with mouse cells have found a decrease in excision repair with prolonged time in culture while Del'Orco and Whittle (1978) and Hasegawa et al. (1984) noted an increase in human fibroblast DNA repair with progressive cell line passages. DNA repair in RTG cells varying from passage 63 to 96 was examined (Figure 13) and no difference in the repair magnitude observed. But the levels of repair noted in cultured RTG cells are substantially higher than found in the rainbow trout primary cells (Figure 22). As the RTG cell line was established in the early 1960's (Wolf and Quimby, 1962) and is commercially available from the American Type Culture Collection only from passage 60-70, it was not possible to investigate changes in DNA repair capacity in earlier passages. Ishikawa et al. (1978) autoradiographically measured in vivo DNA repair in Oryzias latipes, of age 4 to 38 months, the approximate life span of the fish. Their results note no age-associated change in repair ability of ganglion cells.

It was decided that unless the routine RTG DNA repair assay procedure could be altered to yield a response in excess of fifty grains per nucleus, then the monitoring technique would not be worth pursuing, due to its low sensitivity in this cell line. It was found that by conducting the assay at 25 C, incubating the cells with ^{3}HTdR for 6h, and using a 30 day emulsion exposure period, then a 2 to 3-fold increase in grain counts could be achieved (Figure 14), increasing the

assay's sensitivity to desired levels.

d) Using the DNA repair inhibition assay with fish cells

A number of chemicals have been found to inhibit DNA repair (Balachandran and Srinivasan, 1982; Charp and Regan, 1985). Routine application of the repair assay has therefore been complemented by incorporating a test for repair inhibition into the procedure. This test requires an initial treatment of the cells with a genotoxic agent, to damage the DNA, prior to applying a test chemical or extract. The initial damaging agent most often used is ultraviolet light, not a chemical, in order to avoid interactive effects of chemicals while ensuring damage to the DNA. In fish cells the repair inhibition test has limited applicability because, unlike HF cells, thymidine dimers, the primary DNA lesion from ultraviolet light, can be repaired by photoreactivation (Table 2; Regan and Cook, 1967; Mano et al., 1982; Mitani, 1983).

e) DMSO and S9 use in the DNA repair assay

The sparingly soluble nature of most of the test chemicals in tissue culture medium required initially dissolving the chemical in dimethylsulfoxide prior to dilution in medium. Although Novicki et al. (1985) observed no decrease in rat hepatocyte DNA synthesis with DMSO concentrations of 0.5% or less, Nestmann et al. (1985), reported enhanced mutagenicity of hexachloroacetone dissolved in DMSO versus other solvents. The effect of DMSO on DNA repair was therefore examined on ultraviolet light irradiated RTG cells (Figure 15). DNA repair inhibition/cytotoxicity was noted at concentrations in excess of 1% DMSO. The final DMSO

concentration in the culture medium for experiments therefore did not exceed 1%.

Cells maintained in culture for prolonged periods of time lose much of their ability to metabolize test chemicals. As demonstrated in RTG and FHM cells (Diamond and Clark, 1970; Thornton et al., 1982), residual metabolic activity may remain. This enzyme activity may be sufficient to metabolize chemicals, such as AFB_1 (Figure 4) to active forms, but a liver microsomal fraction or S9 is routinely added to chemical dilutions to ensure metabolism of the test chemical or extract. S9 is usually prepared following the procedure of Ames et al. (1975) who used Aroclor 1254 to pre-treat the animals. As the oil-extract or Aroclor 1254 pre-treatment enhances the activity and spectrum of enzymes present and species specific metabolic effects have been observed, a repair comparison was made using rainbow trout and rat S9s pre-treated with Aroclor 1254, and a rainbow trout S9 with oil-extract pre-treatment. The comparison here (Figures 18, 19) noted more repair following AFB_1 metabolism with both fish S9 preparations than with the rat liver S9. Despite the different pre-treatments, little difference was noted between the two fish S9s. These results may reflect rat versus fish S9 differences in reaction kinetics or differences in enzyme activity, as the trout sensitivity to the carcinogenic effects of AFB_1 is felt due to particular metabolic pathways (Sinnhuber et al., 1977; Williams and Buhler, 1983).

f) Screening PAHs for genotoxicity in the DNA repair assay

The DNA repair assay can be used to screen possible

aquatic carcinogens for genotoxic activity. The polycyclic aromatic hydrocarbons were chosen as a representative group of aquatic contaminants some of which, such as 3,4-benzopyrene, 1,2,5,6-dibenzanthracene, and 1,2-benzanthracene, are known carcinogens (National Academy of Sciences, 1972). In contaminated aquatic areas PAHs have been implicated in the etiology of fish tumors (Brown et al., 1973; Black et al., 1980; Malins et al., 1984). Fish S9 has been shown to metabolize benzopyrene and dibenzanthracene to metabolites causing mutagenic activity in the Ames salmonella test (Ahokas et al., 1977; Stegeman, 1978). Benzo(a)pyrene metabolites have also been shown to bind to the DNA of salmonid and pleuronectid fish (Varanasi et al., 1979; Varanasi et al., 1982), but little is known of the repair of these lesions.

Of the four PAHs assayed for genotoxic activity only pyrene is not carcinogenic, while 1,2-benzanthracene is somewhat less carcinogenic than 1,2,5,6-dibenzanthracene and 3,4-benzopyrene (National Academy of Sciences, 1972). As the HF cells have a greater repair response, and hence are more sensitive than RTG cells, the assay was first conducted with both cell lines using the original assay conditions and S9 from Aroclor 1254 treated rainbow trout. DNA repair was only observed in HF cells (Figure 20) treated with BP, indicating that trout S9 can metabolize BP to forms which damage trout DNA. Despite the carcinogenic potential of DBA, no repair activity was noted; perhaps because the solubility of DBA in culture medium was observed to be less than BP. Previously, of these four PAHs, only BP has been shown to cause DNA repair

(Mitchell et al., 1983).

When the assay was conducted using the conditions that enhance the response, repair was observed in RTG cells treated with BP, but not with the other PAHs (Figure 21). Although RTG cells can metabolize BP (Diamond and Clark, 1970; Thornton et al., 1982) the amount of a particular metabolite formed appears insufficient to cause DNA repair. The amount of repair in BP treated cells was greater using S9 from Aroclor 1254 treated rainbow trout. Possibly the S9 from the oil-extract treated rainbow trout metabolized the BP to forms of reduced genotoxic activity. Shelton et al. (1983, 1984) observed a reduced tumor frequency in rainbow trout given Aroclor 1254 prior to AFB_1 treatment and speculated that the exposure to Aroclor may have altered the predominance of metabolic pathways leading to different metabolites.

g) DNA repair assays with fish primary cells and tissue slices

A goal of this research was to develop a technique for directly assessing the genotoxic burden of an aquatic environment. One means of accomplishing this is to determine the level of DNA repair in isolated primary cells of fish collected from different areas. As a step towards this, cells were isolated from the stomach, liver, and intestine of rainbow trout and examined for DNA repair activity following treatment with MNNG, 4NQO, and AFB_1 (Figure 22). Despite employing the assay conditions that enhance the repair response, only modest levels of repair were observed. Similarly Klaunig (1984) and Regan et al.(1983) have noted low

levels of excision repair in fish primary cells.

Such low levels of repair in the rainbow trout primary cells following treatment with potent genotoxic chemicals effectively precluded pursuing this line of endeavor, as the amount of genotoxic activity anticipated in field situations would be much lower.

A second application of the repair technique that was considered was the possibility of distinguishing phylogenetically different cell types on the basis of variation in DNA repair capacity, assuming the human - rodent - fish relationship extended to other species. It has been suggested that the X-cells within the flatfish epidermal papillomas and cod pseudobranchial tumors are parasites, possibly amoebas (Brooks et al., 1969; Alpers et al., 1977; Dawe, 1981). Should the flatfish epithelium surrounding the X-cells exhibit DNA repair to the level of primary cells and the cells thought to be parasites have less repair capacity then one might be able to discriminate the different cells. However, X-cells and epithelial cells examined in sections from tissue pieces treated with 4NQO or MNNG had so few grains that a dose response was not evident. As also observed by Kranz et al. (1980), some surrounding epithelial cells are heavily grained indicating both that there is cell division in this area of the papilloma, and that thymidine is adequately penetrating the tissue. The X-cells cannot therefore be discriminated from the epithelial cells on the basis of DNA repair capacity.

3. Assays for DNA Damage as Tests for Aquatic Genotoxicity

a) Comparison of chromosomal aberration frequency in mammalian and fish cells

The comparatively low amount of DNA repair in fish cells which have approximately the same cellular DNA content as mammalian cells suggested that considerable fish DNA damage may not be repaired. If so, then tests for detecting damage to fish DNA may be more sensitive than repair assays.

Initially a comparative approach was again undertaken using the chromosome aberration test. This test is routinely performed using CHO cells because they have relatively few (2n = 22) large chromosomes, are stable in culture, and, as they have a rapid rate of division, a large number of metaphase spreads for analyzing can be easily obtained. Although RTG cells have been used by Landolt et al. (1984) and Kocan et al. (1985) for studying chromosome aberrations, this cell line has a large and variable number of chromosomes which complicates the analysis. Gold et al. (1979) list chromosome formulae for some 306 North American fish, of which only 5 species have fewer than 30 chromosomes. Because cell lines had not been established for any of these 5 species two lines were initiated from fin and heart tissue of Umbra limi which has 22 large chromosomes (Beamish et al., 1971). As found for the other fish cell lines (Figure 2), the Umbra cells also exhibit considerably less DNA repair (Figure 23). ^{3}HTdR incorporation was also used with dividing cells to determine post-treatment sampling times for chromosome aberrations

(Table 3). A mitotic delay was noted following handling of cells and treatment with 4NQO. The number of cells found to be dividing was dependent on the cell line with comparatively few observed in the Ul-F and HF preparations. The results showed that Ul-H and CHO cells could be sampled 12 to 16h following treatment. Ul-F cells were excluded from the chromosome aberration experiments due to their low mitotic rate. Aberration experiments have previously been successfully done with the HF cells using a post-treatment period of 30h prior to sampling. Later experience indicated that a higher rate of cell division was encouraged by a higher (up to 20%) serum concentration in the culture medium and careful manipulation of the cells in the procedure. Cells growing well in stock cultures also performed better in the experiments. Examination of chromosome aberrations in Ul-H, CHO, and HF cells treated with MNNG or 4NQO indicated a Ul-H dose response at a lower concentration than HF or CHO, while the greatest response occurred in CHO cells (Figure 24). Cytotoxicity in Ul-H cells prevented analyzing for aberrations at higher chemical concentrations. As the CHO cells are the standard cell line for analyzing for aberrations and the Ul-H cells also performed well further tests compared only these two cell lines.

b) Procedural details of the chromosome aberration test

Two assay procedural details were examined. The aberration frequency at particular 4NQO concentrations was fairly consistent at different sampling times (Table 4). A slight decline in aberration frequency was observed in CHO at

the later sampling times but not RTG, perhaps due to a higher level of DNA repair in CHO cells. As noted with DNA repair (Figure 15), final concentrations of DMSO above 1 to 2% were found to be toxic to the fish cells (Table 5). Interestingly a similar effect was not evident with CHO cells.

c) Chromosome aberrations following PAH exposure

A demonstration of the practical use of the aberration test examined the DNA damaging effects of PAH. In parallel with the similar DNA repair experiment (Figures 20, 21), chromosome aberrations were observed only after BP treatment (Figure 27). As previously found in the comparative chromosome aberrations tests the Ul-H dose-response indicates these cells are more sensitive at lower concentrations of the test chemicals, but the CHO cells have a higher aberration frequency.

d) Use of the micronucleus test with fish and mammalian cells

The observed sensitivity of the fish cells to DNA damaging events suggested expanding the scope of the aberration test to surveying fish populations. Both Umbra limi and Umbra pygmaea have been treated in vivo and examined for chromosome aberrations (Mong and Berra, 1979; Hooftman and Vink, 1981). However, very few local fish have a suitable karyotype and the central mudminnow, Umbra limi, is found only in southern Manitoba and Ontario and the northeastern United States (Scott and Crossman, 1973). The micronucleus test which also examines DNA damage, but is not dependent on low numbers of chromosomes was therefore explored as an alternative to the chromosome aberration test. In metaphase

plates damaged DNA may be observed as fragmented chromosomes, but if the cells divide, these fragments aggregate to form so-called micronuclei which can be enumerated to produce dose responses. In vitro comparisons indicated dose responses in HF, CHO, Ul-H, and Ul-F cells following treatment with both 4NQO and MNNG (Figures 25, 26). As the generation of micronuclei is dependent on cell division the higher frequency noted in CHO and Ul-H is likely, in part, related to the faster division of cells in these lines.

Micronuclei have been observed in peripheral blood erythrocytes of the eastern mudminnow Umbra pygmaea following in vivo treatment with ethyl methanesulphonate (Hooftman and de Raat, 1982). In a field survey of inshore and offshore populations of windowpane flounder, winter flounder, and Atlantic mackerel along the United States' eastern seaboard, Longwell et al. (1983) noted a higher incidence of micronuclei in the inshore fish. However, the micronuclei frequencies reported in these papers are quite low. Further complications may also arise from mis-identification of micronuclei, especially in red blood cells. Cytoplasmic inclusions diagnostic of viral erythrocytic necrosis (Smail, 1982) resemble and may be mistaken for micronuclei. Micronucleus-like characters may also result from erythrocyte fragmentation and amitosis which has been observed in Atlantic salmon (Benfey and Sutterlin, 1984) and plaice (Ellis, 1984). Analyzing for micronuclei in cells from tissues such as liver or stomach rather than blood may therefore be preferable.

4. Field Testing the Genotoxicity Assays

a) Overview

Gilbertson (1984) expressed the opinion that fish populations first be surveyed for pathological anomalies prior to developing further laboratory toxicological techniques for exploring a problem. Similarly the work here follows earlier studies in our laboratory which linked a high incidence of epidermal papillomas in flatfish with environmentally degraded areas (Stich et al., 1977a,b). More recent consideration of these tumors has suggested they are parasite infections (Alpers et al., 1977; Dawe, 1981) and a 1984 survey of tumorous fish from the coastal waters of Hokkaido, Japan failed to find a direct association between municipal and industrial discharges with flatfish having the epidermal papillomas (Katsura et al., 1984). However, experiments treating rainbow trout eggs with BP later resulted in the formation of two integumental nodules in alevins, one of which resembled an angioepithelial nodule, a precursor to the flatfish epidermal papillomas (Hose et al., 1984). Elevated 3,4-benzopyrene concentrations were also found in sediments from Sturgeon Bank, near Vancouver (Dunn and Stich, 1976), where Stich and co-workers found the high incidence of flatfish tumors. The Sturgeon Bank area is contaminated with effluent from the Iona Island sewage treatment plant which serves Vancouver. The plant discharges approximately 1,178,000 m^3/d of primary treated sewage which is chlorinated during the summer months. A wide range of chemicals has been identified in the Sturgeon Bank sediments and biota (Bawden et

al., 1973; Koch et al., 1977) and a seasonal variation may be expected due to chlorination effects. Elsewhere a variety of carcinogens such as nitrosamines and polycyclic aromatic hydrocarbons have been isolated from sewage and sewage sludge (Grzybowski et al., 1983; Brewer et al., 1980; Babish et al., 1983; Hopke et al., 1984), genotoxic activity has been found in extracts of fecal material (Stich et al., 1980), and genotoxic activity was observed in extracts of sediments collected near sewage outfalls (Moore et al., 1980; Van Hoof, 1981; Sato et al., 1983). Tumorous growths in salamanders have previously been associated with treated sewage effluent (Rose, 1976; Rose and Harshbarger, 1977). More recently Murchelano and Wolke (1985) found an elevated incidence of liver tumors in fish caught at a raw sewage outfall and Grizzle et al. (1984) found a high prevalence of oral papillomas in fish living in chlorinated effluent from a sewage treatment facility.

b) In vitro genotoxicity testing of the contaminated area

The genotoxicity tests used here with fish and mammalian cells were field tested with sediment samples and fish collections from the Sturgeon Bank - Iona Island sewage treatment plant outfall area. A nearby Spanish Bank area in English Bay was selected as a control site. Virtually no DNA repair activity was noted in either HF or RTG cells treated with extracts of Sturgeon or Spanish Bank sediment extracts (Table 6). Some cytotoxicity was observed in RTG cells treated with Sturgeon Bank sediment extracts but this toxicity was diminished in the presence of S9. The failure to detect

any DNA repair activity may be due to components of the extract which inhibit DNA repair, genotoxic agents not being in high enough concentrations in the extracts, or a lack of sensitivity of the assay.

In the chromosome aberration test some DNA damage was caused in CHO cells by treatment with extracts from sediment samples collected near the outfall (Table 7). A more pronounced effect was noted with Ul-H cells where peak aberration frequencies of 10 to 23% were noted from treatment with extracts from the outfall area. Slight cytotoxicity and DNA damaging activity were noted in both cell lines following treatment with the Spanish Bank #3 sediment extract. This may be due to the observed circulation pattern of the sewage effluent toward English Bay, or contaminant deposition from industrial discharges into the North Arm of the Fraser River influencing this control site (Greater Vancouver Sewerage and Drainage District, 1983). Generally more cytotoxicity was noted with extracts from sediments collected closer to the outfall but the toxic effect is reduced in the presence of S9. Because PAHs, such as BP, require activation to DNA damaging forms, and S9 generally reduced the damaging activity, it is implied that an array of chemicals are present in the extracts, some of which do not require metabolic activation.

c) In vivo genotoxicity assessment of the contaminated area

Despite the difference in DNA damaging activity of the Sturgeon Bank versus Spanish Bank sediments, no increase in micronuclei frequency was found in buccal, blood, or liver cells from starry flounder (Table 8). Possibly the Sturgeon

Bank fish were collected too far out on the Bank. However, DNA damaging activity was detected at the collection site and the muddy nature of the sediments precluded beach seine collections closer to the point of discharge. The fish may be transient, and hence receive only small, perhaps inconsequential exposures to the contaminated sediments. But the contents of the Sturgeon Bank beach seines did show a larger food resource, such as shrimp, and starry flounder are not known for being migratory (Hart, 1973). The starry flounder micronuclei frequencies are also low in comparison to those found in eastern mudminnows treated with methanesulphonate (Hooftman and de Raat, 1983). It may be that the extraction of chemicals from the sediments concentrates the activity to a detectable level which, though not detected in vivo, is still damaging the starry flounder DNA but to a much smaller degree. Possibly starry flounder are not a sensitive indicator species. However, beach seines did not collect substantial numbers of other fish species and examination of the few specimens collected did not indicate a difference in micronuclei frequency.

Future research may expand the applications of these assays, examine a broader range of test chemicals, and other field situations.

SUMMARY

This study's primary objective was to incorporate fish cells and metabolizing enzymes into genotoxicity assays commonly used with mammalian cells in order to:

a) provide assays with increased relevance to fish and the aquatic ecosystem

b) validate mammalian assay results

c) determine fish cell sensitivity to environmentally important genotoxic agents such as PAH

d) develop techniques to directly assess the carcinogenic/genotoxic burden of contaminated environments, such as Sturgeon Bank, near Vancouver.

1. DNA Repair Assay

a) Fish and mammalian cell excision repair was measured in the DNA repair assay following treatment with four genotoxic chemicals. The amount of measured repair varied in the order of human cells > rodent cells > several fish cell lines. Relatively little variation in the repair response was noted among the fish cell lines.

b) Selecting the RTG cell line as being representative of fish cells, it was determined that their repair response was not due to delayed repair of damage, lack of test chemical uptake, or smaller quantity per cell of DNA target.

c) It was found that adequate RTG DNA repair sensitivity could be attained by raising the assay temperature, increasing

the ^{3}HTdR incubation time, and lengthening the ^{3}HTdR degradation period in the autoradiographic workup. Little or no effect on RTG DNA repair was noted from varying the ^{3}HTdR concentration, duration in ADM, ADM serum concentration, and cell line passage.

d) Treatment of HF and RTG cells with a series of PAH indicated that only BP could be activated by fish S9 to result in DNA repair in HF cells and such repair could only be detected in RTG cells when using the assay conditions which enhance the fish cell repair response.

e) In a progression towards *in vivo* measurements, DNA repair was measured in rainbow trout primary cells and starry flounder tissue slices but the measured repair was even less than the small amounts observed in fish cells *in vitro*. Further repair assays were abandoned in favour of the hypothesis that fish cell DNA damage may not be repaired, hence tests examining DNA damage may be considerably more sensitive.

2. Tests for DNA Damage

a) As the RTG cell line is not ideally suited for chromosome work, two cell lines from the central mudminnow, *Umbra limi*, were established.

b) The DNA repair response of U1-F and U1-H lines is comparable to that observed in the other fish cell lines.

c) U1-H cells were more sensitive than CHO or HF cells in chromosome aberration tests but the greatest aberration frequency was observed in CHO cells.

d) Of the PAH series examined only BP metabolized with rainbow trout S9 resulted in chromosome aberrations in Ul-H and CHO cells.

e) Analyzing for DNA damage was extended from examining for chromosome aberrations, to micronuclei as this latter test has wider in vivo applicability.

3. Field Testing the Developed Genotoxicity Tests

a) Prior research had shown an elevated frequency of epidermal papillomas in flatfish collected from Sturgeon Bank, an area contaminated with a variety of chemicals, including PAHs, from a sewage treatment plant (Dunn and Stich, 1976; Stich et al., 1977a,b).

b) Sediments and flatfish were collected from Sturgeon Bank and Spanish Banks, the latter of which was selected as a control site.

c) No DNA repair was observed in HF or RTG cells treated with sediment extracts but some RTG cell cytotoxicity was noted at high concentrations of the Sturgeon Bank sediment extracts.

d) Chromosome aberrations and cytotoxicity were found in CHO and Ul-H cells treated with Sturgeon Bank sediment extracts. With the level of effect generally increasing with extracts from sediments which were collected closer to the sewage treatment plant's discharge point.

e) Examination of Sturgeon Bank starry flounder buccal, liver, and stomach cells for micronuclei failed to demonstrate an in vivo genotoxic effect.

REFERENCES CITED

Ahokas, J., O. Pelkonen, and N. Karki. 1977. Characterization of benzo(a)pyrene hydroxylase of trout liver. Canc. Res. 37: 3737-3743.

Ahokas, J., H. Saarni, D. Nebert, and Pelkonen, O. 1979. The in vitro metabolism and covalent binding of benzo(a)pyrene to DNA catalyzed by trout liver microsomes. Chem.-Biol. Inter. 25: 103-111.

Alink, G. 1982. Genotoxins in waters. Prog. in Clin. and Biol. Res. 109: 261-276.

Alpers, C., B. Cain, M. Meyers, S. Wellings, M. Poore, J. Bagshaw, and C. Dawe. 1977. Pathologic anatomy of pseudobranch tumors in Pacific cod, Gadus macrocephalus. J. Nat. Canc. Inst. 59: 377-398.

Altman, P. and D. Katz. 1976. Cell Biology. Fed. Am. Soc., Bethesda, Maryland. 367-378 pp.

Ames, B., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the Salmonella mammalian-microsome mutagenicity test. Mut. Res. 31: 347-364.

Babish, J., B. Johnson, and D. Lisk. 1983. Mutagenicity of municipal sewage sludges of American cities. Environ. Sci. Technol. 17: 272-277.

Balachandran, R. and A. Srinivasan. 1982. Caffeine inhibits DNA polymerase I from Escherichia coli: studies in vitro. Carcino. 3: 151-153.

Balk, L., J. DePierre, A. Sundvall, and U. Rannug. 1982. Formation of mutagenic metabolites from benzo(a)pyrene and 2-aminoanthracene by the S-9 fraction from the liver of the northern pike (Esox lucius): inducibility with 3-methylcholanthrene and correlation with benzo(a)pyrene monooxygenase activity. Chem. Biol. Inter. 41: 1-13.

Bawden, C., W. Heath, and A. Norton. 1973. A Preliminary Baseline Study of Roberts and Sturgeon Banks. Westwater Research Centre Technical Report No. 1, Univ. Brit. Col.

Beamish, R., M. Merrilees, and E. Crossman. 1971. Karyotypes and DNA values for members of the suborder Esocoidei (Osteichthyes: Salmoniformes). Chromosoma 34: 436-447.

Benfey, T. and A. Sutterlin. 1984. Binucleated red blood cells in the peripheral blood of an Atlantic salmon, Salmo salar L. alevin. J. Fish. Dis. 7: 415-420.

Ben-Ishai, R. and L. Peleg. 1974. Excision-repair in primary cultures of mouse embryo cells and its decline in progressive passages and established cell lines. In Hanawalt, P. and R. Setlow (eds.) Molecular Mechanisms for Repair of DNA, Part B. Plenum Press, New York.

Black, J., E. Evans, J. Harshbarger, and R. Zeigel. 1982. Epizootic neoplasms in fishes from a lake polluted by copper mining wastes. J. Nat. Canc. Inst. 69: 915-926.

Black, J., M. Holmes, B. Paigen, P. Dymerski, and W. Zapisek. 1980. Fish tumor pathology and aromatic hydrocarbon pollution in a Great Lakes estuary. Env. Sci. Res. 16: 559-565.

Bols, N., S. Boliska, D. Dixon, P. Hodson, and K. Kaiser. 1985. The use of fish cell cultures as an indication of contaminant toxicity to fish. Aqu. Tox. 6: 147-155.

Boyland, E. 1969. The correlation of experimental carcinogenesis and cancer in man. Progr. Exptl. Tumor Res. 11: 222-234.

Brewer, W., A. Draper III, and S. Wey. 1980. The detection of dimethylnitrosamine and diethylnitrosamine in municipal sewage sludge applied to agricultural soils. Environ. Pollut. 1B: 37-43.

Bridges, B. 1974. The three-tier approach to mutagenicity screening and the concept of the radiation-equivalent dose. Mut. Res. 26: 335-340.

Brooks, R., G. McArn, and S. Wellings. 1969. Ultrastructural observations on an unidentified cell type found in epidermal tumors of flounders. J. Nat. Canc. Inst. 43: 97-110.

Brown, E., J. Hazdra, L. Keith, I. Greenspan, J. Kwapinski, and P. Beamer. 1973. Frequency of fish tumors found in a polluted watershed as compared to non-polluted Canadian waters. Canc. Res. 33: 189-198.

Bullock, A. 1982. The pathological effects of ultraviolet radiation on the epidermis of teleost fish with reference to the solar radiation effect in higher animals. Proc. Roy. Soc. Edin. 81B: 199-210.

Charp, P. and J. Regan. 1985. Inhibition of DNA repair by trifluoperazine. Biochim. et Biophys. Acta. 824: 34-39.

Chen, F. 1984. Sequence specific binding of tetraols of benzo(a)pyrene-diol-epoxide to DNA in neutral and acidic solution. Carcino. 5: 753-758.

Cleaver, J. and D. Bootsma. 1975. Xeroderma pigmentosum: biochemical and genetic characteristics. Ann. Rev. Genet. 9: 19-38.

Das, S., E. Beneditt, and L. Loeb. 1983. Rapid changes in deoxynucleoside triphosphate pools in mammalian cells treated with mutagens. Biochem. and Biophys. Res. Comm. 114: 458-464.

Dawe, C. 1981. Polyoma tumors in mice and X-cell tumors in fish, viewed through telescope and microscope. In Dawe, C., J. Harshbarger, S. Kondo. T. Sugimura, and S. Takayama (Eds.) Phyletic Approaches to Cancer. Japan Scientific Societies Press, Tokyo, pp. 19-49.

Dell'Orco, R. and W. Whittle. 1978. Unscheduled DNA synthesis in confluent and mitotically arrested populations of aging human diploid fibroblasts. Mech. Ageing Dev. 8: 269-279.

Denizeau, F. and M. Marion. 1984. Cultured rainbow trout and human cells exposed to PCB's in the evaluation of cytotoxicity of aquatic pollutants. Water Res. 18: 247-251.

Diamond, L. and F. Clark. 1970. Comparative studies on the interaction of benzo(a)pyrene with cells derived from poikilothermic and homeothermic vertebrates. I Metabolism of benzo(a)pyrene. J. Nat. Canc. Int. 45: 1005-1012.

Dolara, P., V. Ricci, D. Burrini, and O. Griffini. 1981. Effect of ozonation and chlorination on the mutagenic potential of drinking water. Bull. Environ. Contam. Toxicol. 27: 1-6.

Dunn, B. and A. Hanham. 1983. Unpublished results. British Columbia Cancer Research Center.

Dunn, B. and H. Stich. 1976. Monitoring procedures for chemical carcinogens in coastal waters. J. Fish. Res. Bd. Can. 33: 1040-2046.

Ellis, A. 1984. Bizarre forms of erythrocytes in a specimen of plaice, Pleuronectes platessa L. J. Fish. Dis. 7: 411-414.

Epstein, S. 1974. Environmental determinants of human cancer. Canc. Res. 34: 2425-2435.

Flamm, W. 1974. A tier system approach to mutagen testing. Mut. Res. 26: 329-333.

Fried, J., A. Perez, and B. Clarkson. 1976. Flow cytofluorometric analysis of cell cycle distributions using propidium iodide. J. Cell Biol. 71: 172-181.

Gilbertson, M. 1984. Need for development of epidemiology for chemically induced diseases in fish in Canada. Can. J. Fish Aquat. Sci. 41: 1534-1540.

Gold, J., W. Karel, and M. Strand. 1979. Chromosome Formulae of North American Fishes. The Texas A & M University System, Texas Agricultural Experiment Station, Texas publication MP-1411, 23 pp.

Greater Vancouver Sewerage and Drainage District. 1983. Iona Deep Sea Outfall Feasibility Study. Volume I.

Grizzle, J., P. Melius, and R. Strength. 1984. Papillomas on fish exposed to chlorinated wastewater efluent. J. Nat. Canc. Inst. 73: 1133-1142.

Grzybowski, J., A. Radecki, and G. Rewkowska. 1983. Isolation, identification, and determination of polycyclic aromatic hydrocarbons in sewage. Environ. Sci. Technol. 17: 44-47.

Hart, J. 1973. Pacific Fishes of Canada. Fisheries Research Board of Canada, Bulletin 180, Ottawa, Canada.

Hart, R. and R. Setlow. 1976. DNA repair in late-passage human cells. Mech. Ageing and Dev. 5: 67-77.

Hasegawa, N., F. Hanaoka, and M. Yamada. 1984. Increased unscheduled DNA synthesis in aged human diploid fibroblasts. Mech. Ageing Dev. 25: 297-305.

Hazel, J. and C. Prosser. 1974. Molecular mechanisms of temperature compensation in poikilotherms. Physiol. Rev. 54: 620-677.

Heidelberger, C. 1975. Chemical carcinogenesis. Ann. Rev. Biochem. 44: 79-121.

Hendricks, J., J. Wales, R. Sinnhuber, J. Nixon, P. Loveland, and R. Scanlan. 1980. Rainbow trout (Salmo gairdneri) embryos: a sensitive animal model for experimental carcinogenesis. Fed. Proc. 39: 3222-3229.

Hooftman, R. and G. Vink. 1981. Cytogenetic effects on the eastern mudminnow, Umbra pygmaea, exposed to ethyl methanesulfonate, benzo(a)pyrene, and river water. Ecotox. Env. Safe. 5: 261-269.

Hooftman, R. and W. de Raat. 1982. Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow Umbra pygmaea by ethyl methanesulphonate. Mut. Res. 104: 147-152.

Hopke, P., M. Plewa, P. Stapleton, and D. Weaver. 1984. Comparison of the mutagenicity of sewage sludges. Environ. Sci. Technol. 18: 909-916.

Hose, J., J. Hannah, H. Pufer, and M. Landolt. 1984. Histologic and skeletal abnormalities in benzo(a)pyrene-treated rainbow trout alevins. Arch. Environ. Contam. Toxicol. 13: 675-684.

IARC Monograph, 1973. Evaluation of Carcinogenic Risk of the Chemical to Man. Certain polycyclic aromatic hydrocarbons and heterocyclic compounds. 3: 271 pp.

De La Iglesia, F., R. Lake, and J. Fitzgerald. 1980. Short-term tests for mutagenesis and carcinogenesis in drug toxicology: How and when to test is the question. Drug Metab. Rev. 11: 103-146.

Ishikawa, T., S. Takayama, and T. Kitagawa. 1978. Autoradiographic demonstration of DNA repair synthesis in ganglion cells of aquarium fish at various age in vivo. Virchows Arch. B. Cell. Path. 28: 235-242.

Jack, P. and P. Brookes. 1982. Mechanism for the loss of preferential benzo(a)pyrene binding to the linker DNA of chromatin. Carcino. 3: 341-344.

Kantor, G. and R. Setlow. 1981. Rate and extent of DNA repair in non-dividing human diploid fibroblasts. Canc. Res. 41: 819-825.

Katsura, K., F. Yamazaki, K. Hamada, K. Oishi, T. Harada, and T. Shinkawa. 1984. Geographic distribution and frequency of tumorous fishes collected from the coastal waters of Hokkaido, Japan. Bull. Japan. Soc. Sci. Fish. 50: 979-984.

Klaunig, J. 1984. Establishment of fish hepatocyte cultures for use in in vitro carcinogenicity studies. In Hoover,K. (Ed.) Use of Small Fish Species in Carcinogenicity Testing. Nat. Canc. Inst. Monog. 65. National Cancer Institute, Bethesda, Maryland, pp. 163-173.

Kocan, R., K. Sabo, and M. Landolt. 1985. Cytotoxicity/genotoxicity: the application of cell culture techniques to the measurement of marine sediment pollution. Aqu. Tox. 6: 165-177.

Koch, R., K. Hall, and I. Yesaki. 1977. Toxic Substances in the Wastewaters From a Metropolitan Area. Westwater Research Centre Technical Report No. 12, Univ. Brit. Col.

Kraemer, K., M. Lee, and J. Scotto. 1984. DNA repair protects against cutaneous and internal neoplasia: evidence from xeroderma pigmentosum. Carcino. 5: 511-514.

Kranz, H., N. Peters, G. Bresching, and H. Stich. 1980. On cell kinetics in skin tumours of the Pacific English sole Parophrys vetulus Girard. J. Fish Dis. 3: 125-132.

Kraybill, H.F. 1977. Global distribution of carcinogenic pollutants in water. Ann. N.Y. Acad. Sci, 298: 80-89.

Krishan, A. 1975. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J. Cell Biol. 66: 188-193.

Laishes, B. 1974. The role of metabolic activation of chemical precarcinogens in the induction of DNA damage and repair in mammalian cells. PhD. Thesis, Dept. of Genetics, Univ. Brit. Col.

Landolt, M., R. Kocan, and R. Dexter. 1984. Anaphase aberrations in cultured fish cells as a bioassay of marine sediments. Mar. Env. Res. 14: 497-498.

Lehninger, A. 1975. Biochemistry. pg. 860-861. Worth Publ., New York.

Lo, L. and H. Stich. 1978. The use of short-term tests to measure the preventive action of reducing agents on formation and activation of carcinogenic nitroso compounds. Mut. Res. 57: 57-67.

Longwell, A., D. Perry, J. Hughes, and A. Hebert. 1983. Frequencies of micronuclei in mature and immature erythrocytes of fish as an estimate of chromosome mutation rates - results of field surveys on windowpane flounder, winter flounder, and Atlantic mackerel. Report for the Marine Environmental Quality Committee of the International Council for the Exploration of the Sea.

Lyle, T., R. Royer, G. Daub, and D. Vander Jagt. 1980. Reactivity-selectivity properties of reactions of carcinogenic electrophiles and nucleosides: influence of pH on site selectivity. Chem.-Biol. Inter. 29: 197-207.

Malins, D., B. McCain, D. Brown, S. Chan, M. Myers, J. Landahl, P. Prohaska, A. Friedman, L. Rhodes, D. Burrows, W. Gronlund, and H. Hodgins. 1984. Chemical pollutants in sediments and diseases of bottom-dwelling fish in Puget Sound, Washington. Environ. Sci. Technol. 18: 705-713.

Mano, Y., K. Kator, and N. Egami. 1982. Photoreactivation and excision repair of thymidine dimers in ultraviolet-irradiated cultured fish cells. Radiat. Res. 90: 501-508.

Marion, M. and F. Denizeau. 1983. Rainbow trout and human cells in culture for the evaluation of the toxicity of aquatic pollutants: A study with lead. Aqu. Tox. 3: 47-60.

Melchior, W. and F. Beland. 1984. Preferential reaction of the carcinogen N-acetoxy-2-acetylaminofluorene with satellite DNA. Chem.-Biol. Inter. 49: 177-187.

Meyers, T. and J. Hendricks. 1982. A summary of tissue lesions in aquatic animals induced by controlled exposures to environmental contaminants, chemotherapeutic agents, and potential carcinogens. Mar. Fish. Rev. 44: 1-17.

Meyne, J., L. Lockhart, and F. Arrighi. 1979. Nonrandom distribution of chromosomal aberrations induced by three chemicals. Mutat. Res. 63: 201-209.

Mitani, H. 1983. Lethal and mutagenic effects of radiation and chemicals on cultured fish cells derived from the erythrophoroma of goldfish (Carassius auratus). Mutat. Res. 107: 279-288.

Mitchell, A., D. Casciano, M. Meltz, D. Robinson, R. San, G. Williams, and E. von Halle. 1983. Unscheduled DNA synthesis tests. Mutat. Res. 123: 363-410.

Mong, S. and T. Berra. 1979. The effects of increasing dosages of X-irradiation on the chromosomes of the central mudminnow Umbra limi (Kirtland) (Salmoniformes: Umbridae). J. Fish. Biol. 14: 523-527.

Moore, R., L. Osborne, and R. Davies. 1980. The mutagenic activity in a section of the Sheep River, Alberta receiving a chlorinated sewage effluent. Water Res. 14: 917-920.

Murchelano, R. and R. Wolke. 1985. Epizootic carcinoma in the winter flounder, Pseudopleuronectes americanus. Science 228: 587-589.

National Academy of Sciences. 1972. Particulate Polycyclic Organic Matter. pp. 2-12. National Academy of Sciences Printing and Publishing Office.

Nelson, C. and R. Hites. 1980. Aromatic amines in and near the Buffalo River. Environ. Sci. Technol. 14: 1147-1149.

Nestmann, E., G. Douglas, D. Kowbel, and T. Harrington. 1985. Solvent interactions with test compounds and recommendations for testing to avoid artefacts. Env. Mut. 7: 163-170.

Novicki, D., M. Rosenberg, and G. Michalopoulos. 1985. Inhibition of DNA synthesis by chemical carcinogens in cultures of initiated and normal proliferating rat hepatocytes. Canc. Res. 45: 337-344.

Ornstein, R., and R. Rein. 1979. Nucleic acid base and carcinogen metabolite specificities during intercalative interactions between DNA and 4-nitroquinoline 1-oxide. Chem.-Biol. Inter. 27: 291-311.

Painter, R. 1981. DNA synthesis inhibition in mammalian cells as a test for mutagenic carcinogens. In Stich, H. and R. San (Eds.), Short-term Tests as a Test for Mutagenic Carcinogens. Springer-Verlag, New York, pp. 59-64.

Payne, J. and A. Rahimtula. 1981. Water chlorination as a source of aquatic environmental mutagens. In Khan, M. and R. Stanton (Eds.), Toxicology of Halogenated Hydrocarbons: Health and Ecological Effects. Permagon Press, New York, pp. 209-221.

Payne, J. and I. Martins. 1980. Monitoring for mutagenic compounds in the marine environment. Rapp. P.-V. Reun. Cons. Int. Explor. Mer. 179: 292-298.

Perin-Roussel, O., A. Croisy, B. Ekert, and F. Zajdela. 1984. The metabolic activation of dibenzo[a,e]fluoranthene in vitro. Evidence that its bay-region and pseudo-bay-region diol-epoxides react preferentially with guanosine. Canc. Lett. 22: 289-298.

Peters, N. and B. Watermann. 1979. Three types of skin papillomas of flatfishes and their causes. Mar. Ecol. Prog. Ser. 1: 269-276.

Peters, N., G. Peters, H. Stich, A. Acton, and G. Bresching. 1978. On differences in skin tumors of Pacific and Atlantic flatfish. J. Fish. Dis. 1: 3-25.

Pierce, K., B. McCain, and S. Wellings. 1978. Pathology of hepatomas and other liver abnormalities in English sole (Parophrys vetulus) from the Duwamish River estuary, Seattle, Washington. J. Nat. Cancer Inst. 60: 1445-1453.

Plumb, J. and K. Wolf. 1971. Fish cell growth rates: Quantitative comparison of RTG-2 cell growth at 5-25 C. In Vitro 7: 42-45.

Regan, J. and J. Cook. 1967. Photoreactivation in an established vertebrate cell line. Proc. Nat. Acad. Sci. 58: 2274-2279.

Regan, J., R. Snyder, A. Francis, and B. Olla. 1983. Excision repair of ultraviolet- and chemically-induced damage in the DNA of fibroblasts derived from two closely related species of marine fishes. Aqu. Tox. 4: 181-188.

Rogers, A. 1979. Techniques of Autoradiography. 3rd Ed. Elsevier, Amsterdam.

Rose, F. 1976. Tumorous growths of the tiger salamander, Ambystoma tigrinum, associated with treated sewage effluent. Prog. Exp. Tum. Res. 20: 251-262.

Rose, F. and J. Harshbarger. 1977. Neoplastic and possibly related skin lesions in neotenic tiger salamanders from a sewage lagoon. Science 196: 315-317.

Rosen, P. 1980. Ultraviolet carcinogenesis in the teleost fish, Poecilia formosa. Med. Hypoth. 6: 1057-1061.

San, R. 1976. DNA repair synthesis in cultured human fibroblasts as a bioassay for chemical carcinogens. PhD Thesis, Dept. of Genetics, Univ. Brit. Col.

Sato, T., T. Momma, Y. Ose, T. Ishikawa, and K. Kato. 1983. Mutagenicity of Nagara River sediment. Mutat. Res. 118: 257-267.

Saxena, J. and D.J. Schwartz. 1979. Mutagens in wastewaters renovated by advanced wastewater treatment. Bull. Environm. Contam. Toxicol. 22: 319-326.

Scott, W. and E. Crossman. 1973. Freshwater Fishes of Canada. Bulletin 184, Fisheries Research Board of Canada, Ottawa, Canada.

Scovassi, A., R. Wicker, and U. Bertazzoni. 1979. A phylogenetic study on vertabrate mitochondrial DNA polymerase. Eur. J. Biochem. 100: 491-496.

Shelton, D., J. Hendricks, R. Coulombe, and G. Bailey. 1984. Effect of dose on the inhibition of carcinogenesis/ mutagenesis by aroclor 1254 in rainbow trout fed aflatoxin B_1. J. Tox. Env. Hlth. 13: 649-657.

Shelton, D., R. Coulombe, C. Pereira, J. Casteel, and J. Hendricks. 1983. Inhibitory effect of aroclor 1254 on aflatoxin-inititated carcinogenesis in rainbow trout and mutagenesis using a Salmonella/trout hepatic activation system. Aqu. Tox. 3: 229-238.

Sinnhuber, R., J. Hendricks, J. Wales, and G. Putnam. 1977. Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. Ann. N.Y. Acad. Sci,. 298: 389-408.

Smail, D. 1982. Viral erythrocytic necrosis in fish: a review. Proc. Roy. Soc. Edinburgh 81B: 169-176.

Smith, C., R. Peck, R. Klauda, and J. McLaren. 1979. Hepatomas in Atlantic tomcod Microgadus tomcod (Walbaum) collected in the Hudson River estuary in New York. J. Fish. Dis. 2: 313-319.

Sober, H., R. Harte, and E. Sober. 1968. CRC Handbook of Biochemistry. pp H: 45-48, 54-58. The Chemical Rubber Co., Cleveland, Ohio.

Stegeman, J. 1978. Metabolic activation of foreign compounds to toxic and mutagenic derivatives by fish. Abstract. Symposium on carcinogenic polynuclear aromatic hydrocarbons in the marine environment. EPA. Gulf Breeze, Florida.

Stich, H., A. Acton, and C. Forrester. 1976. Fish tumors and sublethal effects of pollutants. J. Fish. Res. Board Can. 33: 1993-2001.

Stich, H., A. Acton, B. Dunn, K. Oishi, F. Yamazaki, T. Harada, G. Peters, and N. Peters. 1977a. Geographic variations in tumor prevalence among marine fish populations. Int. J. Cancer 20: 780-791.

Stich, H., A. Acton, K. Oishi, F. Yamazaki, T. Harada, T. Hibino, and H. Moser. 1977b. Systematic collaborative studies on neoplasms in marine animals as related to the environment. Ann. N.Y. Acad. Sci. 298: 374-388.

Stich, H. and R. San. 1970. DNA repair and chromatid anomalies in mammalian cells exposed to 4-nitroquinoline 1-oxide. Mut. Res. 10: 389-404.

Stich, H. and R. San. 1981. Short-term Tests for Chemical Carcinogens. Springer-Verlag, New York, 518 pp.

Stich, H., B. Dunn, A. Acton, F. Yamazaki, K. Oishi, and T. Harada. Unpublished results.

Stich, H., L. Wei, and R. Whiting. 1979. Enhancement of the chromosome-damaging action of ascorbate by transition metals. Canc. Res. 39: 4145-4151.

Stich, H., R. San, and P. Lam. 1977c. Possible use of short-term tests for carcinogens in experimental epidemiology. Nat. Cancer Inst. Monogr. 47: 81-85.

Stich, H., W. Stich, and A. Acton. 1980. Mutagenicity of fecal extracts from carnivorous and herbivorous animals. Mut. Res. 78: 105-112.

Stott, W. and R. Sinnhuber. 1978. Trout hepatic enzyme activation of aflatoxin B_1 in a mutagen assay system and the inhibitory effect of PCB's. Bull. Environm. Contam. Toxicol. 19: 35-41.

Thornton, S., L. Diamond, and W. Baird. 1982. Metabolism of benzo(a)pyrene by fish cells in culture. J. Tox. Env. Hlth. 10: 157-167.

De Torrengo, M. and R. Brenner. 1976. Influence of environmental temperature on the fatty acid desaturation and elongation activity of fish (Pimelodus maculatus) liver microsomes. Biochim. et Biophys. Acta. 424: 36-44.

Van Hoof, F. and J. Verheyden. 1981. Mutagenic activity in the River Meuse in Belgium. The Sci. Tot. Environ. 20: 15-22.

Varanasi, U. and D. Gmur. 1980. Metabolic activation and covalent binding of benzo(a)pyrene to deoxyribonucleic acid catalyzed by liver enzymes of marine fish. Biochem. Pharmacol. 29: 753-761.

Varanasi, U., D. Gmur, and M. Krahn. 1979. Metabolism and subsequent binding of benzo(a)pyrene to DNA in pleuronectid and salmonid fish. In A. Bjorseth and A. Dennis (Eds.) Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects. Battelle Press, Columbus, Ohio.

Varanasi, U., M. Nishimoto, W. Reichert, and J. Stein. 1982. Metabolism and subsequent covalent binding of benzo(a)pyrene to macromolecules in gonads and liver of ripe English sole (Parophrys vetulus). Xeno. 12: 417-425.

Varanasi, U., J. Stein, and T. Hom. 1981. Covalent binding of benzo(a)pyrene to DNA in fish liver. Biochem. Biophys. Res. Comm. 103: 780-787.

Warren, P. and H. Stich. 1975. Reduced DNA repair capacity and increased cytotoxicity following split doses of the mutagen 4-nitroquinoline-1-oxide in cultured human cells. Mut. Res. 28: 285-293.

Warters, R., B. Lyons, D. Ridinger, and L. Dethlefsen. 1985. DNA damage repair in quiescent murine mammary carcinoma cells in culture. Biochim. et Biophys. Acta. 824: 357-364.

Werner, D., D. Hadjioloo, and H. Hershey. 1981. Non-random distribution of N-methyl-N-nitrosourea sensitive sites in a eukaryotic genome. Chem.-Biol. Inter. 37: 279-287.

Williams, D. and D. Buhler. 1983. Purified form of cytochrome P-450 from rainbow trout with high activity toward conversion of aflatoxin B_1 to aflatoxin B_1-2,3-epoxide. Canc. Res. 43: 4752-4756.

Wolf, K. 1979. Cold-blooded vertebrate cell and tissue culture. In Jakoby, W. and I. Pastan (Eds.) Methods in Enzymology: Volume LVIII Cell Culture. Academic Press, New York, 1979, pp.466-477.

Wolf, K. and J. Mann. 1980. Poikilotherm vertebrate cell lines and viruses: a current listing for fishes. In Vitro 16: 168-179.

Wolf, K. and M. Quimby. 1962. Established eurythermic line of fish cells in vitro. Science 135: 1065-1066.

Wolf, K. and M. Quimby. 1976. Primary monolayer culture of fish cells initiated from minced tissues. TCA Manual 4: 445-448.

Woodhead, A., R. Setlow, and E. Grist. 1980. DNA repair and longevity in three species of cold-blooded vertebrates. Exp. Geront. 15: 301-304.

Yu, F. 1983. Preferential binding of aflatoxin B_1 to the transcriptionally active regions of rat liver nucleolar chromatin in vivo and in vitro. Carcino. 4: 889-893.

APPENDIX I

Publications from the Thesis Material

a) Accepted

1. Walton, D., A. Acton, and H. Stich. 1983. DNA repair synthesis in cultured mammalian and fish cells following exposure to chemical mutagens. Mut. Res. 124: 153-161.

2. Walton, D., A. Acton, and H. Stich. 1984. DNA repair synthesis following exposure to chemical mutagens in primary liver, stomach, and intestinal cells isolated from rainbow trout. Canc. Res. 44: 1120-1121.

3. Walton, D., A. Acton, and H. Stich. 1984. Comparison of DNA-repair synthesis, chromosome aberrations and induction of micronuclei in cultured human fibroblast, Chinese hamster ovary and central mudminnow (Umbra limi) cells exposed to chemical mutagens. Mut. Res. 129: 1129-136.

4. Walton, D., A. Acton, and H. Stich. 1985. Increased response of the rainbow trout gonad cell unscheduled DNA repair assay. Bull. Environ. Contam. Toxicol. 34: 340-348.

5. Walton, D. In press. In vitro fish cell DNA breakage and repair assays for detecting mutagenic activity. Aquatic Toxicity Conference Proceedings. Can. Tech. Rep. Fish. Aquat. Sci.

b) Submitted

1. Walton, D., A. Acton, and H. Stich. DNA repair synthesis in cultured fish and human cells exposed to fish S9-activated aromatic hydrocarbons.

2. Walton, D. The use of fish cells in short-term mutagenicity tests.

APPENDIX II

List of Abbreviations

ADM	-	arginine-deficient medium
AFB_1	-	aflatoxin B_1
BA	-	1,2-benzanthracene
BP	-	3,4-benzopyrene
CH	-	chum salmon heart cell line
CHO	-	Chinese hamster ovary cell line
DBA	-	1,2,5,6-dibenzanthracene
DMSO	-	dimethylsulfoxide
FHM	-	fathead minnow cell line
HF	-	human fibroblast cell line
^{3}HTdR	-	[methyl-^{3}H]-thymidine
MEM	-	Eagle's minimal essential medium
MNNG	-	N-methyl-N'-nitro-N-nitrosoguanidine
NA2AAF	-	N-acetoxy-2-acetylaminoflourene
4NQO	-	4-nitroquinoline 1-oxide
PAH	-	polycyclic aromatic hydrocarbon
PY	-	pyrene
RTG	-	rainbow trout gonad cell line
RTO	-	rainbow trout ovary cell line
S9	-	liver microsomal fraction, 9000g supernatant
Ul-F	-	*Umbra limi* fin cell line
Ul-H	-	*Umbra limi* heart cell line

APPENDIX III

Theoretical Aspects and Statistical Analysis of the DNA Repair and Breakage Assays

1. DNA Repair Assay

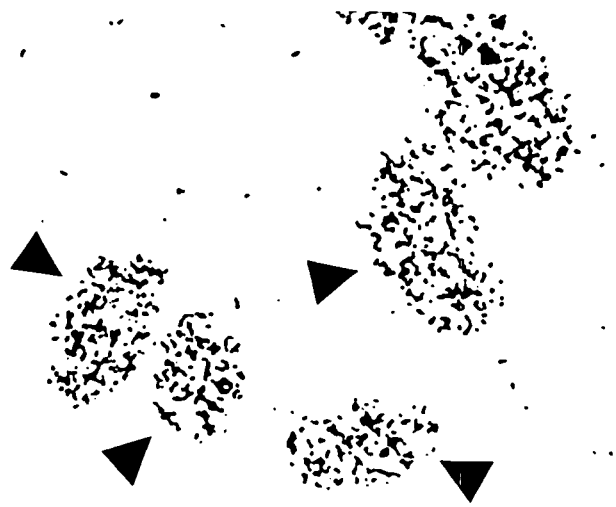
The DNA repair assay measures the amount of nucleotide excision repair by the incorporation of ^{3}HTdR in damaged DNA. In the autoradiographic workup beta-particles emitted from ^{3}HTdR react with the nuclear track emulsion coating on the slides. The silver grains formed over the nuclei (Figure 28) are enumerated and the data graphed to produce dose response curves. In the grain counting process the background grain count, determined from an area equivalent in size and adjacent to each nucleus, was subtracted to give a net grain count which was plotted.

The factors noted produce a 2 to 3 - fold increase of the nuclear grain counts (Results, section A-1-c) also increased negative control (no chemical) and background grain counts. These increases in negative control counts were far overshadowed by the increases in experimental counts (eg. 1 --> 3 vs. 20 --> 60). Background grain counts were also found to increase but the increase was minimized by thorough rinsing of ^{3}HTdR from the coverslips and using a low cell density on the coverslips. However, occasionally, despite these efforts, background grain counts in some experiments, for unknown reasons, were sufficiently high that enumeration was aborted.

FIGURE 28

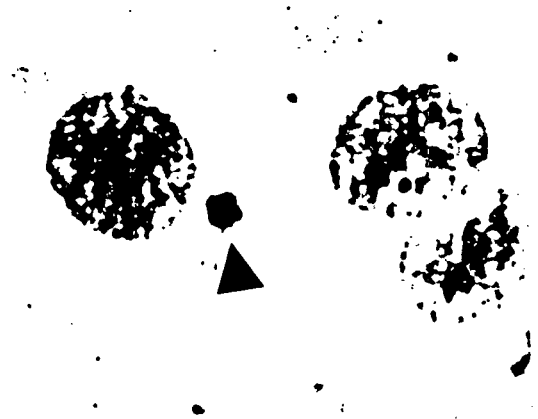
Assay preparations showing (A) DNA repair in rainbow trout gonad cells (arrows indicate nuclei), (B) chromosome aberrations in an Umbra limi heart cell (arrow indicates chromatid breads), and (C) a micronucleus in an Umbra limi heart cell (arrow indicates a micronucleus).

A



B

C



Initial experiments scored 60 nuclei while later experiments scored 30, as little improvement in the data was found with the additional counts. Only nuclei of the same staining intensity and size were scored for a particular slide.

Comparison of positive (4NQO-exposed) controls from *in vitro* sediment extract exposure experiments (Results, section C-1-a) indicated a 20-25% variation in counts between experiments with HF or RTG cells. Negative control grain counts (no chemical) are noted in the Figures. Typically these net grain counts were less than 3 with a standard deviation of 1 to 2.

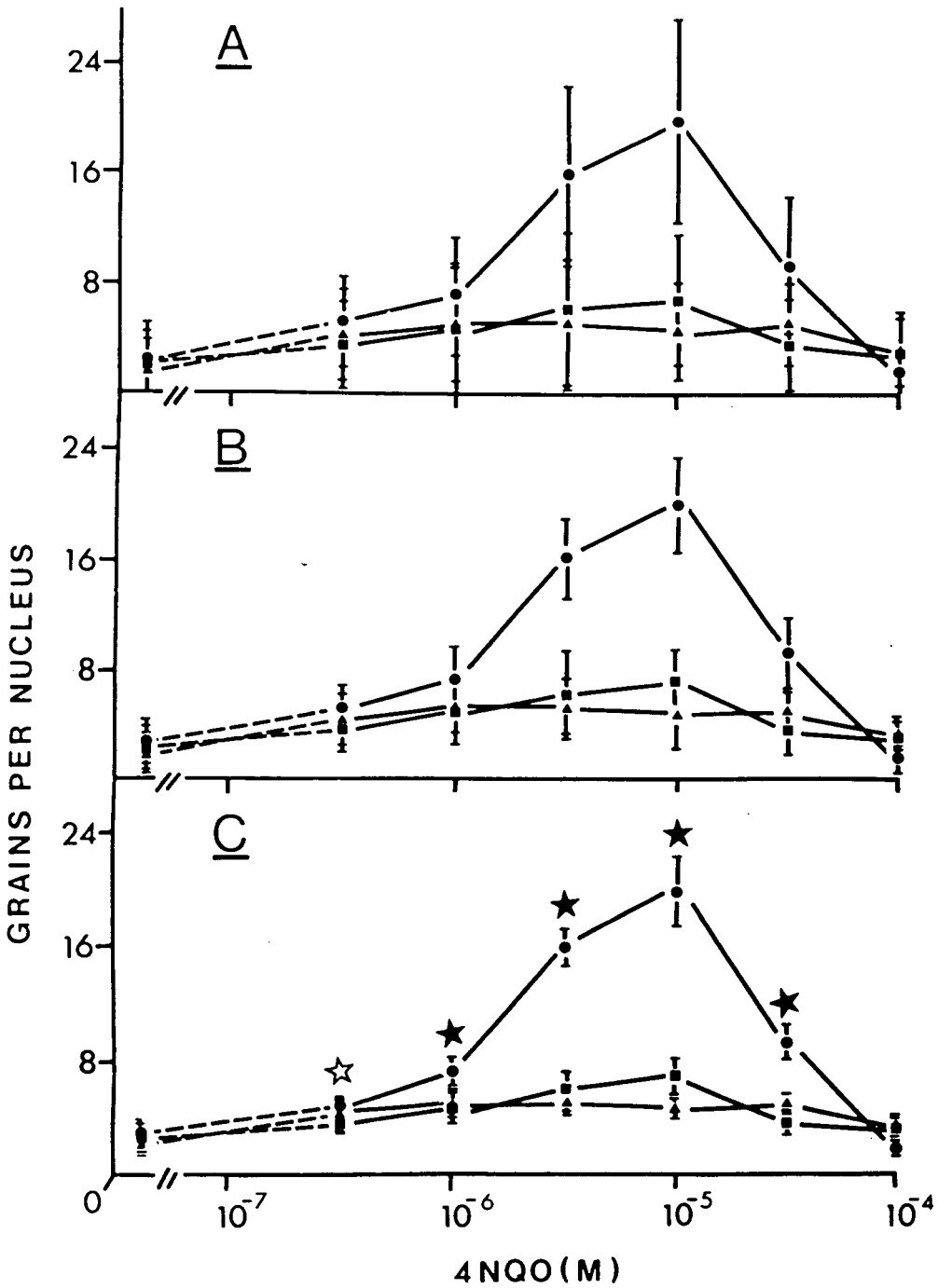
Historically discrimination of data significance has relied on observing a dose response. More recently statistical tests such as the t-test have been used (Klaunig, 1984).

For the DNA repair results presented here, data significance was viewed in three ways. Firstly, the gross appearance of the dose response was appraised to see if a response was obviously present. Secondly, counts of 5-10 grains per nucleus were viewed as representing only nominal amounts of repair and little credance was placed on data significance. Grain counts in excess of 10 grains per nucleus were deemed significant. Thirdly, the number of data points in excess of the 5-10 level were considered. If only 1 or 2 data points were above this level again little credance was placed on data significance.

Statistical significance associated with the 5-10 and

FIGURE 29

Plots of the data from Figure 22 B showing variation on the mean as represented with (A) standard deviations, (B) 99% confidence intervals, and (C) standard errors. The stars indicate statistical significance as calculated using a two-tailed t-test (- p 0.10; - p < 0.001).


A
B
C
24
16
8
0
GRAINS PER NUCLEUS
10^{-7}
10^{-6}
10^{-5}
10^{-4}
4NQO(M)

greater than 10 levels of grain counts were considered (Figure 29) using a two-tailed t-test. 0.01 and < 0.001 levels of significance were associated with grain counts in the 5-10 range while the counts in excess of 10 grains per nucleus had less than the 0.001 level of significance.

Graphical representation of variation on the mean was provided using standard deviations although confidence intervals and standard errors were also considered (Figure 29). As the standard errors proved to be so small that they were difficult to plot and standard deviations were a calculator function, hence easily and immediately available, the latter was chosen.

2. Chromosome Aberration Test

This test detects unrepaired or misrepaired damage to the DNA. At approximately 20-24 h post-exposure to a test chemical the mitotic-spindle poison, colchicine, was added to the culture medium for a 4 h period. Cell division cannot therefore proceed and is arrested in metaphase. The resultant spreads of chromosomes were examined for damage which, in CHO and U1-H cells, was noted to most often be chromatid breaks and exchanges (Figure 28). Although not statistically proven, the frequency of exchanges in U1-H cells was observed to be considerably lower than in CHO cells.

Historically data significance has been assessed by observing a dose-response. Variation between experiments occurs and, from positive (4NQO-exposed) controls in the chromosome aberration experiments with the sediment extracts

(Results, section C-1-b), was found to be 10-15% with CHO cells and 20-25% with the U1-H cells. For the research reported here negative control (no chemical) levels of aberrations were typically less than 3%. As with the DNA repair data, significance of the chromosome aberration data was first assessed by discriminating a dose-response. Nominal amounts of breakage were ascribed to levels of 5-10% breakage while truely significant amounts of damage were attributed to incidences in excess of 10%. Added credance in data significance was felt by having more than 1 or 2 data points in excess of the 5-10% level of breakage.

3. Micronucleus Test

The micronucleus test is a gross measure of DNA breakage hence is somewhat of a proxy for chromosome aberrations. In comparison to the aberration test the micronucleus assay detects DNA strand breakage but not exchanges. Micronuclei are therefore pieces of broken DNA material which do not migrate to the poles in mitosis, rather they remain as small balls of nuclear material in the daughter cells (Figure 28). In the experiments, micronuclei were distinguished by size, shape, staining relative to the nucleus, and by focusing up and down through the cell.

As demonstrated (Results, section B-1-a) the <u>in vitro</u> micronucleus dose-response was influenced by mitotic delay following chemical exposure, the rate of cell division, and the time until sampling after exposure. The significance of the <u>in vitro</u> data can only be judged by observation of

a dose-response and discrimination of the incidence of micronuclei at a particular chemical concentration relative to the negative control (no chemical). For these experiments (Figures 25, 26) a strong dose-response with a 5-10% incidence of micronuclei was deemed significant. When evaluating the *in vivo* data (Table 8) the t-test can be applied.

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